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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 3 September 2004 with an application for Letters Patent number 535131 made by FAC8CELL PTY LIMITED.

I further certify that pursuant to a claim under Section 88 of the Patents Act 1953, a direction was given that the application proceed in the name of OLGA GARKAVENKO; ROBERT BARTLETT ELLIOTT;ALFRED VASCONCELLOS;DWAINE EMERICH; CHRIS THANOS.

Dated 5 July 2005

PRIORITY DOCUMENT
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535131

NEW ZEALAND

PATENTS ACT 1953

PROVISIONAL SPECIFICATION

"LIVER CELL TRANSPLANTATION"

We, FAC8CELL PTY LIMITED, a company duly incorporated under the laws of Australia of 160 Greenhill Road, Parkside, South Australia 5063, Australia, do hereby declare this invention to be described in the following statement:

TECHNICAL FIELD

The invention relates to the use of secretory cells for the treatment of diseases associated with a deficiency in a secreted factor and/or a deficiency in organ or tissue function. More particularly but not exclusively it relates to methods of isolating and culturing secretory cells, enhancing the survival of secretory cells, and implanting secretory cells, for the treatment of such diseases, and in particular, to the isolation, characterization, culturing, and use of liver cells.

Using the methods described herein, isolated secretory cells retain for extended periods the capability of mediating cell-specific functions. Therefore, isolated secretory cells may have a wide range of applications, including, but not limited to, their use to augment, replace and/or reconstitute a functionally deficient secretory organ by, for example, implantation.

BACKGROUND OF THE INVENTION

Many diseases, deficiencies and conditions can be treated by supplying to the patient one or more biologically active factors produced and/or secreted by living cells. In many cases, these factors can restore or compensate for the impairment or loss of organ or tissue function. Examples of diseases or conditions whose etiologies include loss of secretory organ or tissue function include, but are not limited to, diabetes, wherein the production of insulin by pancreatic islets β-cells is impaired or lost; hypoparathyroidism, wherein the loss of production of parathyroid hormone causes serum calcium levels to drop resulting in severe muscular tetany; hypothyroidism and cretin dwarfism, both due to thyroid hormone deficiency; hypophysial dwarfism, due to pituitary growth hormone deficiency; Lesch-Nyhan Syndrome, due to hypoxanthine-guanine phosphoribosyltransferase deficiency; fulminant hepatic failure, due to the hepatotrophic factor deficiency; hemophilia A, wherein a deficiency in the production and/or secretion of the blood clotting factor FactorVIII (FVIII) can lead to severe bleeding episodes; Parkinsonism, wherein dopamine production is diminished; and anemia, which is characterized by the loss of production of red blood cells secondary to a deficiency in erythropoietin.

In some diseases, conditions or deficiencies whose etiologies lie in the loss or diminishment of a single biologically active factor, replacement or replenishment of said factor may be therapeutically effective. For example, administration of insulin to diabetics can significantly improve the control of serum glucose and delay the onset of complications associated with elevated serum glucose.

However, the impairment or loss of organ or tissue function may result in the loss of multiple metabolic functions. For example, it has been reported that in fulminant hepatic failure, liver tissue is rendered incapable of removing toxins, excreting the products of cell metabolism, and secreting essential factors, such as albumin and Factor VIII (Bontempo, *et al.*, Blood, 69, pp. 1721-1724 (1987)).

In many diseases or conditions, the affected organ or tissue is one which normally functions in a manner responsive to the physiological state, by, for example, responding to fluctuations in the levels of specific metabolites and/or physiologically important substances, thereby maintaining homeostasis. For example, pancreatic islet β-cells normally modulate production of insulin in response to fluctuations in serum glucose. Traditional therapeutic approaches to the treatment of such diseases cannot compensate for the responsiveness of the normal tissue to these fluctuations. For example, an accepted treatment for diabetes includes daily injections of insulin. This regimen cannot compensate for the rapid, transient fluctuations in serum glucose levels produced by, for example, strenuous exercise or ingestion of food. It has been suggested that failure to provide such attuned responsiveness to the physiological state may lead to complications of the disease state.

Many other diseases are, likewise, characterized by a deficiency in a biologically active factor that cannot easily be supplemented by injections or longer-term, controlled release therapies. Still other diseases, while not characterized by substance deficiencies, can be treated with biologically active factors normally made and secreted by cells. Thus, trophic and growth factors may be used to prevent neurodegenerative conditions, such as Huntington's and

Alzheimer's diseases, and adrenal chromaffin cells which secrete catecholamines and enkephalins may be used to treat pain.

Still other diseases are characterised by impairment or loss of organ or tissue function. Accordingly, many investigators have attempted to reconstitute organ or tissue function by transplanting whole organs or organ tissue to provide secreted products or effect metabolic functions. For example, liver transplantation is the established therapy for end-stage liver disease, as described by Starzl, *et al.,* N. Eng. J. Med. 321:1014-1022 (1989). In another example, patients with hemophilia A have undergone liver transplantation as a result of liver failure resulting from hepatitis acquired from the blood derived factor VIII. In these instances, there has been a complete cure of the hemophilia. In a further example, diseases characterised by the impairment or loss of organ or tissue function may result in the accumulation of deleterious factors which are normally metabolised by or as a result of the organ or tissue. Such diseases, efficiencies and conditions may be treated by removing from or reducing in the patient said deleterious factors by, for example, orgmentation replacement and/or reconstitution of organ or tissue function. Transplantation can provide dramatic benefits, but is limited in its application by the scarcity of organs available for transplantation. For example, it has been reported that more than 25,000 people die each year in the United States of liver disease (Murphy, SL. Deaths: final data for 1998. Natl. Vital Stats. Rep. 2000; 48:1-105), and 11% of those listed for transplantation in 2001 died while waiting for an organ (Annual report of the U.S. Organ Procurement and Transplant Network and the Scientific Registry of Transplant Recipients, 2003).

In general, the patient must undergo immunosuppression or immunomodulation in order to avert immunological rejection of the transplant, which results in loss of transplant function and eventual necrosis of the transplanted organ or tissue. However, immunosuppressive or immunomodulatory therapy generally impairs the patient's overall immunological defences, which may increase susceptibility to the risks of a variety of serious complications, including nephrotoxicity, neurotoxicity, hypertension, increased susceptibility to infection and osteoporosis. Moreover, this approach is not always effective in altering the course

and incidence of rejection episodes. Typically, the transplant must remain functional for a long period of time, even for the remainder of the patient's lifetime. It is both undesirable and expensive to maintain a patient in an immunosuppressed or immunomodulated state for a substantial period of time.

Transplanted cells provide the potential for treating various diseases because the cells can provide factors to replace or supplement natural factors which, due to their insufficiency or absence, cause disease. Cell implantation therapy has an advantage over traditional factor-supplementation therapy regimens because the transplanted cells are responsive to the physiologic state, by, for example, response to fluctuations in the levels of specific metabolites and/or physiologically important substances in the recipient. The release of therapeutic factors from the transplanted cells may be properly regulated provided the transplanted cells have the necessary receptors and ability to respond to endogenous regulators.

Patients having disease as a result of the loss or deficiency of secreted factors, such as proteins, enzymes, hormones, neurotransmitters, growth factors or other physiological substances are considered to be among those who would achieve significant benefits from transplant therapy. For example, implantation of pancreatic islet cells could provide insulin as needed to a diabetic.

Transplanted cells also provide the potential for treating various diseases characterised by the impairment or loss of organ or tissue function, as the transplanted cells can augment, restore or compensate for loss or impairment of organ or tissue function. Cell implantation therapy has an advantage over traditional organ transplantation therapies in that the availability of cells suitable for implantation is not limited as are suitable organs from cadaveric or live organ donors.

Because cells which are implanted may be foreign to the host it is necessary to prevent the host immune system from attacking and thereby causing the death of the implanted cells. Various immunosuppressive or immunomodulatory methods to protect the implanted cells by attenuating the host immune response are possible. However, these methods suffer the disadvantages associated with immunosuppression or immunomodulation therapies used in conjunction with organ

transplantation. There is the further disadvantage of potentially producing adverse effects on the transplanted cells, for example, impairing their engraftment and/or function and/or reducing their secretory responses.

A desirable alternative to such attenuation procedures involves the isolation of the implanted cells from effectors of the recipient's immune response by, for example, implantation within a physical barrier which will allow diffusion of nutrients, waste materials, and secreted products, but block the cellular and molecular effectors of immunological rejection. A variety of methods which isolate implanted cells from the immune system have been explored. These include the use of extravascular diffusion chambers, intravascular diffusion chambers, intravascular ultrafiltration chambers, and implantation of microencapsulated or macroencapsulated cells. Such methods have the potential to obviate the need to subject the patient to immunosuppression or immunomodulation therapy.

However, none of these approaches have been satisfactory for providing long-term transplant function. A method of delivering biologically appropriate quantities of needed factors or providing other needed physiological or functions for an extended period of time is still unavailable and would be very advantageous to those in need of long-term treatment.

It would therefore be advantageous to have a method for enhancing the survival and/or maintenance of function of cells capable of secreting one or more factors in a form suitable for implantation in a patient in need of said one or more factors.

It would furthermore or alternatively be advantageous to have a method for enhancing the survival of cells capable of providing metabolic functions for an extended period of time in a form suitable for implantation in a patient in need of said metabolic function.

It is an object of the invention to provide methods of preparing cells capable of secreting one or more factors and/or providing one or more metabolic and/or physiological functions in a form suitable for implantation in a patient in need of said one or more factors and/or one or more metabolic and/or physiological functions, or to provide those in need of said methods with useful choice.

It is a further or alternative object of the invention to provide methods of preparing preparations, aggregates or other devices for implantation of secretory liver cells, a method of treatment of hemophilia and/or other blood-clotting disease or disorders, and/or a means to aid treatment of hemophilia and/or other blood-clotting diseases or disorders which has improvements to, or provides an alternative from, the abovementioned methods and/or means.

STATEMENTS OF THE INVENTION

According to a first aspect of the invention there is provided **a method of preparing a composition comprising one or more secretory liver cells** capable upon implantation into a recipient of the production and/or secretion of one or more factors, said method including or comprising the steps of:

isolation of one or more secretory liver cells,

culturing of said one or more secretory liver cells in media supplemented with allogeneic serum,

recovery of said one or more secretory liver cells, and

formation of said composition.

Preferably said one or more secretory liver cells are neonatal secretory liver cells.

According to a second aspect of the invention there is provided **a method of preparing a composition comprising one or more liver cells** capable upon implantation into a recipient of the provision of one or more metabolic and/or physiologic functions to said recipient, said method including or comprising the steps of:

isolation of one or more liver cells,

culturing of said one or more liver cells in media supplemented with allogeneic serum,

recovery of said one or more liver cells, and

formation of said composition.

In another aspect the present invention provides **a method of preparing a composition comprising one or more neonatal liver cells** capable upon

implantation into a recipient of the production and/or secretion of one or more factors and/or the provision of one or more metabolic and/or physiologic functions to a recipient in need thereof, said method including or comprising the steps of:

isolation of one or more neonatal liver cells,

culturing of said one or more neonatal liver cells in media supplemented with allogeneic serum,

recovery of said one or more neonatal liver cells, and

formation of said composition.

Preferably said production, secretion, and/or provision is long-term production, secretion, and/or provision.

According to another aspect of the invention there is provided **a method of preparing one or more associations of at least one liver cell associated with at least one other liver cell and/or companion cell** capable upon implantation into a recipient of the production and/or secretion of one or more factors and/or the provision of one or more metabolic and/or physiologic functions to a recipient in need thereof, said method including or comprising the steps of:

isolation of one or more liver cells,

isolation of said at least one other liver cell and/or companion cell,

culturing of said one or more liver cells in media supplemented with allogeneic serum,

recovery of said one or more liver cells, and

formation of said one or more associations with at least one liver cell and/or companion cell.

Preferably said formation of said one or more associations is promoted and/or achieved by culturing said one or more liver cell in allogeneic serum, more preferably together with said at least one other liver cell and/or companion cell.

In another aspect the present invention provides **a method of preparing a composition comprising at least one liver cell associated with at least one other liver cell and/or companion cell** capable upon implantation into a recipient of the production and/or secretion of one or more factors and/or the provision of one or

more metabolic and/or physiologic functions to a recipient in need thereof, said method including or comprising the steps of:

culturing of said one or more liver cells in media supplemented with allogeneic serum,

recovery of said one or more liver cells,

association of said one or more liver cells with said at least one other liver cell and/or companion cell, and

formation of said composition.

Preferably said association is promoted and/or achieved by culturing said liver cell in allogeneic serum, more preferably together with said at least one other liver cell and/or companion cell.

According to a further aspect of the invention there is provided **a method of preparing one or more aggregates of one or more secretory liver cells** capable upon implantation into a recipient of the production and/or secretion of one or more factors, said method including or comprising the steps of:

isolation of one or more secretory liver cells,

culturing of said one or more cells in media supplemented with allogeneic serum,

formation of said one or more aggregates,

wherein said allogeneic serum is capable of providing a growth and/or trophic and/or mitogenic function to said secretory liver cells.

Preferably said production and/or secretion is long-term production and/or secretion.

Preferably, said culturing is in collagen-coated culture vessels.

Preferably, the secretory liver cells are human secretory liver cells.

Preferably the secretory liver cells are neonatal secretory liver cells.

In one embodiment, the secretory liver cells are hepatocytes, preferably neonatal hepatocytes.

In one embodiment, the hepatocytes are isolated from cell cultures such as those available from Cell Dynamics LLC (Smyrna, Georgia, USA).

In an alternative embodiment, the secretory liver cells are gall bladder cells, preferably gall bladder endothelial and/or epithelial cells.

In a further alternative embodiment, the secretory liver cells are selected from one or more of non-parenchymal liver cells, sinusoid cells, liver vessel cells, liver endothelial cells, gall bladder cells or ductal epithelial cells from the gall bladder.

In one embodiment, one or more of the factors is a blood clotting factor.

Preferably the blood clotting factor is Factor VIII.

Alternatively the blood clotting factor is Factor IX.

More preferably the blood clotting factors are both Factor VIII and IX.

In one embodiment, said aggregate additionally comprises one or more companion cells, wherein said companion cell is capable of providing a growth and/or trophic and/or mitogenic function to said secretory liver cells.

Preferably, said companion cells are capable of enhancing the activity, stability, bioavailability, and/or efficacy of said one or more factors.

In one embodiment, said secretory liver cells are derived from the same species as the recipient.

Preferably said companion cells are derived from the same species as the recipient. Alternatively, said companion cells are derived from a species other than that of the recipient.

Preferably, when one or the one or more factors is Factor VIII, said secretory liver cells and/or said companion cells are capable of producing von Willebrand factor, more preferably von Willebrand factor associated and/or complexed with said Factor VIII.

More preferably said von Willebrand factor is that of the same species as the recipient.

In another embodiment, the one or more factors is a growth and/or differentiation factor.

Preferably the growth and/or differentiation factor is selected from growth hormone and analogues thereof, insulin like growth factor and analogues thereof, hepatocyte growth factor and analogues thereof, or fibroblast growth factor and analogues thereof.

In another embodiment, the one or more factors is an enzyme.

Preferably the culturing step is over a time period longer than 7 days.

Preferably the isolation of the secretory liver cells is followed by purification of the said cells.

Preferably the isolation and/or purification of the secretory liver cells comprise or include the steps of:

surgical removal,

collagenase digestion,

washing and culturing of the cells.

Preferably the digestion involves Liberase® H.

Preferably the isolation is as herein described in respect of liver cells, including gall bladder cells, or liver endothelial cells.

Preferably the method includes the step of virological and microbiological testing and/or monitoring of the aggregates and/or components thereof.

Preferably or alternatively the method includes a prestep (before isolation of said cells) of virological monitoring and/or testing of one or preferably both of the secretory liver cells and companion cells.

Preferably the method includes additionally or alternatively a prestep of virological monitoring and/or testing of the donor subjects.

Preferably the secretory liver cells and companion cells derive from the same species.

Preferably the donor subjects are monitored and/or tested for infectious agents.

Preferably the step of the formation of the aggregates involves the preservations of the original characteristics of the secretory liver cells.

According to another aspect of the invention there is provided **a method of preparing one or more aggregates and/or one or more associations of one or more secretory liver cells and one or more companion cells** capable upon implantation into a recipient of the production and/or secretion of one or more factors, said method including or comprising the steps of:

isolation of one or more secretory liver cells,

isolation of one or more companion cells,

culturing of said one or more secretory liver cells,

culturing of said one or more companion cells, and

formation of said one or more aggregates and/or one or more associations,

wherein said companion cells are capable of providing a growth and/or trophic and/or mitogenic function to said secretory liver cells.

Preferably said production and/or secretion is long-term production and/or secretion.

Preferably said secretory liver cells are cultured together with said companion cells.

Preferably said secretory liver cells are derived from the same species as the recipient.

Preferably the secretory liver cells are neonatal secretory liver cells.

Preferably, the secretory liver cells are human secretory liver cells.

In one embodiment, the secretory liver cells are hepatocytes, preferably neonatal hepatocytes.

Preferably the hepatocytes are isolated from cell cultures such as those available from Cell Dynamics LLC (Smyrna, Georgia, USA).

In an alternative embodiment, the secretory liver cells are gall bladder cells, preferably gall bladder endothelial and/or epithelial cells.

In a further alternative embodiment, the secretory liver cells are selected from one or more of non-parenchymal liver cells, sinusoid cells, liver vein cells, liver endothelial cells.

Preferably the companion cells are selected from one or more of Sertoli cells, fibroblasts, growth-arrested fibroblasts, ovarian cells analogous to Sertoli cells, genetically modified cells, gall bladder cells, and endothelial cells including endothelial cells from blood vessels or umbilical cord.

In one embodiment, one or more of the factors is a blood clotting factor.

Preferably the blood clotting factor is Factor VIII.

More preferably the blood clotting factors are both Factor VIII and IX.

In another embodiment, the one or more factors is a growth and/or differentiation factor.

Preferably the growth and/or differentiation factor is selected from growth hormone and analogues thereof, insulin-like growth factor and analogues thereof, hepatocyte growth factor and analogues thereof, or fibroblast growth factor and analogues thereof.

According to another aspect of the invention there is provided **a method of preparing one or more aggregates of one or more liver cells** capable upon implantation of the provision of one or more metabolic and/or physiologic functions to a recipient in need thereof, said method including or comprising the steps of:

isolation of one or more liver cells,

culturing of said one or more liver cells in media supplemented with allogeneic serum, and formation of said one or more aggregates.

Preferably said liver cells are neonatal liver cells.

Preferably said provision is long-term provision.

According to yet another aspect of the invention there is provided **a method of preparing one or more aggregates and/or one or more associations or one or more liver cells and one or more companion cells** capable upon implantation of the provision of one or more metabolic and/or physiologic functions to a recipient in need thereof, said method including or comprising the steps of:

isolation of one or more liver cells,

isolation or one or more companion cells,

culturing of said one or more liver cells,

culturing of said one or more companion cells, and

formation of said one or more aggregates and/or one or more associations,

wherein said companion cells are capable of providing a growth and/or trophic and/or mitogenic function to said liver cells.

Preferably said liver cells are neonatal liver cells.

Preferably said provision is long-term provision.

According to a further aspect of the invention there is provided **a composition comprising one or more liver cells** capable upon implantation into a

recipient of the long-term production and/or secretion of one or more factors and/or the long-term provision of one or more metabolic and/or physiologic functions.

Preferably said liver cells are neonatal liver cells.

Preferably said one or more liver cells are isolated and/or prepared using a method which comprises contacting said cells with a composition comprising allogeneic serum.

According to further aspects the present invention provides **compositions, associations, and/or aggregates** prepared substantially according to any of the above methods.

According to a further aspect of the invention there is provided **a method of treating a patient suffering from or predisposed to a disease or condition associated with a deficiency in or absence of a secreted factor** comprising or including the implantation or other administration of one or more compositions, associations or aggregates of one or more secretory liver cells to the patient.

Preferably said one or more secretory liver cells are isolated and/or prepared using a method which comprises contacting said cells with a composition comprising allogeneic serum.

Preferably said one or more compositions, associations or aggregates are prepared using a method which comprises contacting said one or more secretory liver cells with a composition comprising allogeneic serum.

Preferably said compositions, associations or aggregates are prepared substantially according to any of the methods herein disclosed.

In one embodiment said compositions, associations or aggregates comprise one or more companion cells.

Preferably, said disease or condition is chronic liver insufficiency, liver failure, liver disease, or alcoholic liver disease.

In one embodiment, said insufficiency, failure or disease is caused by infection, preferably infection with hepatitis A or B virus.

According to a further aspect of the invention there is provided **a method of treating a patient suffering from or predisposed to a blood clotting disease or condition** comprising or including the implantation or other administration of one

or more compositions, associations or aggregates of one or more secretory liver cells to the patient.

Preferably said one or more secretory liver cells are isolated and/or prepared using a method which comprises contacting said cells with a composition comprising allogeneic serum.

Preferably said one or more compositions, associations or aggregates are prepared using a method which comprises contacting said one or more secretory liver cells with a composition comprising allogeneic serum.

Preferably said compositions, associations or aggregates are prepared substantially according to any of the methods herein disclosed.

In one embodiment said compositions, associations or aggregates comprise one or more companion cells.

According to a further aspect of the invention there is provided **a method of treating a patient suffering from or predisposed to hemophilia and/or a blood-clotting disease or disorder** comprising or including the implantation or other administration of one or more compositions, associations or aggregates of one or more secretory liver cells to the patient.

Preferably said hemophilia is hemophilia A.

Preferably said one or more secretory liver cells are isolated and/or prepared using a method which comprises contacting said cells with a composition comprising allogeneic serum.

Preferably said one or more compositions, associations or aggregates are prepared using a method which comprises contacting said one or more secretory liver cells with a composition comprising allogeneic serum.

Preferably said compositions, associations or aggregates are prepared substantially according to any of the methods herein disclosed.

Preferably said one or more secretory liver cells are human secretory liver cells.

Preferably said one or more secretory liver cells are neonatal secretory liver cells.

In one embodiment said compositions, associations or aggregates comprise one or more companion cells.

Preferably the implantation or administration of the one or more compositions, associations or aggregates may be by one or more of the following:

encapsulation of said one or more compositions, associations or aggregates in a suitable biocompatible material (more preferably a suitable alginate);

confinement into a suitable device (more preferably a vascularized tube for example);

encapsulation in matrix preparations including preparations comprising gelatin, collagen, and/or natural carbohydrate polymers;

confinement into a plasma thrombin clot including allogeneic plasma clots produced with allogeneic thrombin.

According to another aspect of the invention there is provided **a method of administering to a recipient a need thereof a blood clotting factor** wherein said blood clotting factor is complexed and/or associated with one or more factors capable of enhancing the activity, stability, bioavailability, and/or efficacy of said blood clotting factor, wherein the method includes or comprises the implantation or other administration of one or more compositions, associations or aggregates of one or more secretory liver cells to the patient.

Preferably said one or more secretory liver cells are isolated and/or prepared using a method which comprises contacting said cells with a composition comprising allogeneic serum.

Preferably said one or more compositions, associations or aggregates are prepared using a method which comprises contacting said one or more secretory liver cells with a composition comprising allogeneic serum.

Preferably said compositions, associations or aggregates are prepared substantially according to any of the methods herein disclosed.

Preferably said one or more secretory liver cells are human secretory liver cells.

Preferably said one or more secretory liver cells are neonatal secretory liver cells.

In one embodiment said compositions, associations or aggregates comprise one or more companion cells.

Preferably said blood clotting factor is Factor VIII, more preferably said blood clotting factor is Factor VIII and said one or more factors capable of enhancing the activity, stability, bioavailability, and/or efficacy of said blood clotting factor is von Willebrand factor.

According to a further aspect of the invention there is provided **a method of treatment of a patient suffering from or predisposed to a disease or condition associated with a deficiency in a metabolic and/or physiologic function** comprising or including the implantation or other administration of one or more compositions, associations or aggregates of one or more liver cells to the patient.

Preferably said one or more liver cells are isolated and/or prepared using a method which comprises contacting said cells with a composition comprising allogeneic serum.

Preferably said one or more compositions, associations or aggregates are prepared using a method which comprises contacting said one or more liver cells with a composition comprising allogeneic serum.

Preferably said compositions, associations or aggregates are prepared substantially according to any of the methods herein disclosed.

Preferably said one or more liver cells are human liver cells.

Preferably said one or more liver cells are neonatal liver cells.

In one embodiment said compositions, associations or aggregates comprise one or more companion cells.

Preferably, said disease or condition is chronic liver insufficiency, liver failure, liver disease, or alcoholic liver disease.

In one embodiment, said insufficiency, failure or disease is caused by infection, preferably infection with hepatitis A or B virus.

According to yet a further aspect of the invention there is provided **a device for implantation** into a recipient suffering from or predisposed to a disease associated with a deficiency in or absence of a secreted factor, the device incorporating one or more compositions, associations or aggregates of one or more

secretory liver cells, said one or more compositions, associations or aggregates being, or possessing the characteristics of, the compositions, associations or aggregates previously described.

In one embodiment, said aggregates additionally comprise one or more companion cells.

According to yet a further aspect of the invention there is provided **a device for implantation** into a recipient suffering from or predisposed to hemophilia and/or blood-clotting disease or disorder, the device incorporating one or more compositions, associations or aggregates of one or more secretory liver cells, said one or more compositions, associations or aggregates being, or possessing the characteristics of, the compositions, associations or aggregates previously described.

In one embodiment, said aggregates additionally comprise one or more companion cells.

Preferably said one or more secretory liver cells are human secretory liver cells.

Preferably the device incorporating the one or more compositions, associations or aggregates may be one of the following:

a suitable biocompatible material as a capsule (more preferably of a suitable alginate);

a vascularized tube or chamber, more preferably a TheraCyte™ device available from TheraCyte, Inc., CA;

a matrix preparation including a preparation of gelatin, collagen, and/or natural carbohydrate polymers;

a plasma thrombin clot including allogeneic plasma clots produced with allogeneic thrombin.

According to a further aspect of the invention there is provided **a method of preparing compositions, associations or aggregates of secretory liver cells and companion cells** prepared substantially as herein described with or without reference to the examples or figures.

According to a further aspect of the invention there is provided **a method of preparing compositions, associations or aggregates of secretory liver cells** prepared substantially as herein described with or without reference to the examples or figures.

According to a further aspect of the invention there is provided **a method of preparing compositions, associations or aggregates of hepatocytes and companion cells** prepared substantially as herein described with or without reference to the examples or figures.

According to a further aspect of the present invention there is provided **a method of preparing compositions, associations or aggregates of liver cells** prepared substantially as herein described with or without reference to the examples or figures.

According to yet a further aspect of the present invention there is provided **a method of preparing compositions or aggregates of neonatal liver cells** prepared substantially as herein described with or without reference to the examples or figures.

According to a further aspect of the invention there is provided an **aggregate of human secretory cells and human companion cells** substantially as described herein and with reference to any one or more of the examples or figures.

In another aspect the present invention provides **a method of isolating liver cells**, said method comprising or including the steps of:

communution of isolated liver,

removal of blood and/or blood cells,

digestion with collagenase,

termination of digestion,

washing and harvesting of cells by centrifugation.

Preferably removal of blood and/or blood cells is achieved by washing.

Preferably digestion is with 0.1% Liberase® H, more preferably with 0.1% Liberase® H at 37° for about 10 minutes, still more preferably digestion is with 0.1% Liberase® H at 37° for about 10 minutes and performed three times.

Preferably termination of digestion is with a composition comprising allogeneic serum, more preferably with a composition comprising about 10% allogeneic serum.

Preferably washing and harvesting of cells is performed by centrifugation at about 50g for about five minutes, more preferably said washing and harvesting is performed more than once.

In another embodiment, the method comprises the additional step following communution of incubation in cold media supplemented with 10% allogeneic serum.

Preferably said incubation is for about 24 hours.

Preferably said media is DMEM/F12.

More preferably said incubation is with five volumes with respect to liver of media.

In another embodiment, the method comprises the additional steps following centrifugation of removal of supernatant, centrifugation at about 500g for about 15 minutes, and washing and harvesting of cells by centrifugation, wherein said cells are non-parenchymal cells.

In another aspect the present invention provides **a method of isolating gall bladder cells**, said method comprising or including the steps of:

communution of isolated gall bladder,

removal of bile and/or blood and/or blood cells,

digestion with collagenase,

termination of digestion,

washing and harvesting of cells by centrifugation.

Preferably removal of bile and/or blood and/or blood cells is achieved by washing.

Preferably digestion is with 0.2% Liberase® H, more preferably with 0.2% Liberase® H at 37° for about 30 minutes.

Preferably termination of digestion is with a composition comprising allogeneic serum, more preferably with a composition comprising about 10% allogeneic serum.

Preferably washing and harvesting of cells is performed by centrifugation at about 500g for about ten minutes, more preferably said washing and harvesting is performed more than once.

In another aspect the present invention provides **a method of isolating liver endothelial cells**, said method comprising or including the steps of:

communution of isolated liver vessel(s),

removal of blood and/or blood cells,

digestion with collagenase,

termination of digestion,

washing and harvesting of cells by centrifugation.

Preferably removal of blood and/or blood cells is achieved by washing.

Preferably digestion is with 0.2% Liberase® H, more preferably with 0.2% Liberase® H at 37° for about 30 minutes.

Preferably termination of digestion is with a composition comprising allogeneic serum, more preferably with a composition comprising about 10% allogeneic serum.

Preferably washing and harvesting of cells is performed by centrifugation at about 500g for about ten minutes, more preferably said washing and harvesting is performed more than once.

This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

DESCRIPTION OF THE FIGURES

Figure 1 depicts a graph of factor VIII and albumin production by a hepatocyte preparation as described in Example 1 herein.

Figure 2 depicts a graph of albumin production by hepatocyte preparations as described in Example 2.1 herein, wherein ARF+FBS indicates

hepatocytes grown in the presence of mitomycin arrested fibroblasts (ARF) in media supplemented with 10% fetal bovine serum (FBS), ARF+PS indicates hepatocytes grown in the presence of ARF in media supplemented with 10% porcine serum (PS), ARF NO S indicates hepatocytes grown in the presence of ARF in serum-free media, COLL+PS indicates hepatocytes grown in collagen coated flasks in media supplemented with 10% PS, C+PS+F indicates hepatocytes grown in collagen (C) coated flasks in media supplemented with 5% PS and 5% fibroblast-conditioned growth media (F), C+PS+S indicates hepatocytes grown in collagen coated flasks in media supplemented with 5% PS and 5% Sertoli-conditioned growth media (S).

Figure 3 depicts a graph presenting albumin production by fibroblasts in growth medium supplemented with porcine serum (F+PS), hepatocytes in growth media supplemented with porcine serum (Hep +PS), hepatocytes in media supplemented with foetal bovine serum (Hep+FBS), as described in Example 2.4 herein.

Figure 4 depicts a graph presenting cell counts of hepatocytes isolated by our standard procedure and by the cold ischemia method, as described in Example 4 herein.

Figure 5 depicts a graph presenting albumin production by hepatocytes isolated by our standard procedure and by the cold ischemia method, as described in Example 4 herein.

Figure 6 depicts a graph presenting factor VIII production by hepatocytes isolated by our standard procedure and by the cold ischemia method, and by gall bladder cells, as described in Example 4 herein.

Figure 7 shows albumin release from hepatocytes incorporated into a TheraCyte device maintained *in vitro*, as described in Example 6 herein.

Figure 8 shows Factor VIII release from hepatocytes incorporated into a TheraCyte device maintained *in vitro*, as described in Example 6 herein.

DETAILED DESCRIPTION

The methods, compositions, devices, and aggregates of the instant invention are useful for long-term, physiologically-responsive provision of a wide range of biologically active factors to an individual in need thereof and/or to provide needed metabolic and/or physiologic functions to an individual in need thereof. Biologically active factors used in the methods and aggregates of the invention include a wide variety of molecules normally secreted by the liver. For example, Factor VIII (FVIII) can be delivered to a Type A hemophiliac, or α1-antitrypsin can be delivered to a patient with α1-antitrypsin deficiency.

The methods, compositions, devices, and aggregates described herein can also be used to restore or augment vital liver-mediated metabolic and/or physiologic functions, such as the removal of toxins or harmful metabolites (e.g., cholesterol) from the bloodstream, by liver cells such as, for example, hepatocytes or non-parenchymal liver cells, or gall bladder epithelial or endothelial cells. The methods compositions, devices, and aggregates of the invention make possible the implantation of cells without the concomitant need to immunosuppress the recipient for the duration of treatment. Through use of the methods, compositions, devices, and aggregates of this invention, homeostasis of particular substances and/or metabolic and/or physiologic function can be restored and maintained for extended periods of time.

Loss of or reduction in liver function is responsible for a great number of diseases, conditions and deficiencies. For example, inborn errors of metabolism relating to the liver individually are rare but collectively are common. The biological basis of the majority of inborn errors of metabolism relating to the liver is single gene defects, which result in abnormalities in the synthesis or catabolism of proteins, carbohydrates, or fats. Most inborn errors of metabolism relating to the

liver are due to a defect in a biological factor, such as an enzyme or protein, which leads to a block in a metabolic pathway. Pathophysiological effects most commonly result from toxic accumulations of substrates before the block, accumulation of intermediates from alternative metabolic pathways, and/or defects in energy production and utilization caused by a deficiency of products beyond the block.

For example, hemophilia A results from an inherited deficiency of clotting factor VIII, normally produced by the liver. When less that 1% of normal factor VIII activity exists in the blood, severe bleeding episodes in response to minimal trauma occur.

Hemophilia affects about 1/10,000 live births around the world. About 1/3rd of these cases are in the severe category.

The established treatment is replacement by injection of the missing FVIII. Isolated FVIII was originally derived in semipurified form from blood, and was thereby subject to the problems associated with blood-derived products, such as being a potential source of infectious agents, such as HIV, or Hepatitis B and C. Blood-derived FVIII has in part been replaced by recombinant factor VIII.

Ideally, FVIII is given prophylactically, but therapy is very expensive (about $100,000/ year). Furthermore, neutralizing antibodies may be generated in the patient, inhibiting the activity of the injected factor.

Occasionally, patients with hemophilia A have undergone liver transplantation as a result of liver failure resulting from hepatitis acquired from the blood derived FVIII. In these instances, there has been a complete cure of the Hemophilia.

Examples of diseases or conditions suitable for treatment with methods, compositions, devices and aggregates of the present invention include diseases or conditions characterised by liver cell death or dysfunction including but not limited to chronic liver insufficiency, liver failure, or liver disease, for example, that caused by infection such as infection with hepatitis A or B virus, and alcoholic liver disease.

Other examples of diseases or conditions suitable for treatment with methods, compositions, devices and aggregates of the present invention include the

following: diseases characterised by cell death or dysfunction including but not limited to endocrine diseases, Diabetes, congenital adrenal hyperplasia and adrenal insufficiency, Hypothyroid diseases, Hypoparathyroid diseases, Hypogonadism, Diabetes insipidus, growth hormone deficiency; disorders of imino acid metabolism including but not limited to Hyperprolinaemia, Hydroxyprolinaemia; disorders of tryptophan metabolism including but not limited to Xanthurenic aciduria, Hydroxykynureninuria, Carcinoid syndrome, Kynureninuria, Dihydropleridine reductase deficiency; disorders of the gamma glutamyl cycle including but not limited to Glutamic acid decarboxylase deficiency, Glutamate dehydrogenase deficiency, 5-oxoprolinuria (pyroglutamic aciduria), Glutathionaemia, *y*-glutamyl-cysteine synthetase deficiency; Organic Acidurias including but not limited to: Methylmalonic acidaemia, Propionic acidaemia including but not limited to: Methylcrotonyl glycinuria, Methyl–hydroxybutiric aciduria, Hydroxy–methylglutaric aciduria, Succinyl-CoA; 3-ketoacid CoA-transferase deficiency, Lactic and pyruvic acidosis, Threonine sensitive ketoacidosis including ketothiolase deficiency, Non-ketotic dicarboxylicaciduria, Caeruloplasmin deficiency (Kinnear-Wilson's disease), C'l-esterase inhibitor deficiency, transferrinaemial, 1-antitrypsin deficiency, Amyloidosis, Afibrinogenaemia; deficiencies in Blood clotting factors; Enzymopathies including but not limited to: Acutalasia, Glucose-6-phosphate dehydrogenase deficiency, Pseudocholinesterase deficiency, Hypophosphatasia; Immunoglobulin (antibody) deficiency syndrome including but not limited to: Congenital hypogammaglobulinaemias, X-linked recessive with lymphopenia and thymic alymphoplasia, X-linked recessive without lymphopenia, sporadic congenital with or without lymphopenia, Ataxia-telangiectasia syndrome, Wiskott-Aldrich syndrome, Dysgammaglobulinaemia including transient, Dysgammaglobulinaemia including congenital, Dysgammaglobulinaemia including acquired, Adenosine deaminase deficiency, Purine nucleotide phosphorylase deficiency; disorders of amino acids including: Hyper-alaninaemia; Inborn Errors of Protein Metabolism including but not limited to: Analbuminaemia, Idipathic hypoproteinaemia, Asymptomatic protein deficiencies; disorders of red cells including but not limited to: Pyruvate kinase deficiency, Hexokinase (HK)

deficiency, Phosphohexose (PHI) isomerase deficiency, Triose phosphate isomerase (TPI) deficiency, 2,3 Diphossphoglycerate mutase deficiency, Phosphoglycerate kinase (PGK) deficiency, Adenosine triphosphatase (ATP-ase) deficiency, Glucose-6-phosphate dehydrogenase (G-6-PD) deficiency, Reduced glutathione deficiency, Glutathione reductase deficiency, Glutathionine peroxidase deficiency, Glutathione synthesis defect, Methaemoglobin reductase; Galactosaemia, Acatalasia, Arginino-succinic aciduria; disorders of pigment metabolism including but not limited to: Albinism, Porphyria including Congenital erythropoietic prophyria, Erythropoietic protoporphyria, Hepatic porphyria including: Acute intermittent (Swedish type) porphyria, Hereditary coproporphyria, Mixed or variegate oporphyria, Cutaneous hepatic porpyria (tarda), Hyperbilirubinaemia, Unconjugated hyperbilirubinaemia including: Glucose-6-phosphate dehydrogenase deficiency, Crigler-Najjar syndrome, Conjugated hyperbilirubinaemia including: Dubin Johnson syndrome, Rotor's disease, Methaemoglobinaemias, Haemochromatosis, Disorder of melanin pigmentation; Disorders of Purine Metabolism including but not limited to: Xanthinuria, Hyperuricaemia, Gout, Lesch-Nyhan syndrome, Secondary uricaemia; Disorders of carbohydrate metabolism including but not limited to: Galactosaemia, Galactokinase deficiency, Uridine diphosphate galctose-4-epimerase deficiency, Hereditary fructose intolerance (HFI), Fructose-1, 6-diphosphatase deficiency; Disorders of polysaccharide metabolism-glycogen storage diseases (Glycogenoses) including but not limited to: Type I – Von Gierke's disease (Hepatorenal GSD), Type Ib (Hepatorenal GSD), Type II – Pompe's disease (Generalised GSD), Type III – Cori's disease (limit dextrinosis), Type IV – Andersen's disease (Amylopectinosis), Type V – McArdle's disease, Type VI – Hers disease, Type VII – Tauri's disease, Type VIII – Huijing's disease, Glycogen synthetase deficiency (Aglycogenosis), Muscle phosphohexosisomermase deficiency; Disorders of mucopolysaccharide metabolism including but not limited to: Type I – Hurler syndrome, Scheie syndrome, Hurler syndrome, Type II – Hunter syndrome, Type III – Sanfilippo Syndrome (A, B and C), Type IV – Morquio-Brailsford syndrome (A and B), Type VI – Maroteaux- Lamy syndrome, Type VII – Glucuronidase deficiency; Glycorprotein storage diseases including but not limited to

Mannosidosis, Fucidosis, Aspartylgucoaminuria; disorders of amino acid metabolism including: disorders of aromatic amino acid metabolism including but not limited to: Phenykletonuria, Dihydropteridine reductase deficiency, Methylmandelic aciduria, Tyrosinaemia, tyrosyluria, tyrosinosis, Richner-Hanhard syndrome, Albinism, Xanthism, Hermansky-Pudlak syndrome, Chediak-Higashi disease, Cross syndrome, Dysautonomia (Riley-Day syndrome); disorders of metabolism of sulphur containing amino acids (cystine; cystathione; homocystine; methionine) including Homocystinuria, Homocystinuria with methylmalonic aciduria, $N^{5.10}$Methyleneterahydrofolate reductase deficiency, Cystathioninuria, Sulphite oxidase deficiency including β-mercaptolactase-cysteine disulphiduria, Cystinuria, Cystinosis, Hypermethioninaemia, Methionine malabsorption (Oast-house syndrome); disorders associated with hyperammonaemia including but not limited to: Argininosuccinicaciduria, Citullinaemia, Hyperornithinaemia, Argininaemia, N-Acetylglutamate synthetase deficiency, Carbamyl phosphate synthetase deficiency, Ornithine transcarbamylase deficiency, Ornithinaemia; disorders of lysine metabolism including but not limited to Hyperlysinaemia, Periodic hyperlysinaemia with hyperammonaemia, Saccharopinuria, Pipecolic acidaemia, *a*-Ketoadipic aciduria, Glutaric aciduria, Crotonic acidaemia, Hydroxylysinaemia, Hydroxylsinuria, Ehlers-Danlos syndrome (type VI); disorders of branched-chain amino acid metabolism including but not limited: Maple syrup urine disease, Hyperleucine-isoleucinaemia, Methylmalonic acidaema, Propionic acidaemia, Methylcrotonylglycinuria, *a*-Methyl-hydroxybutiric aciduria, Methyl glutaconic aciduria, Hydroxyl-methylglutaric aciduria, Isovalericacidaemia, Hypervalinaemia; disorders of histidine metabolism including but not limited to: Carnosinaemia, Urocanic aciduria, disorders of folic acid metabolism (cyclohydrolase and formiminotransferase deficiency), Glutamate formiminotransferase deficiency; Disorders of Glycine Metabolism including but not limited to: Hyperglycinaemia, D-Glyceric acidaemia, Oxalosis, Sarcosinaemia, Trimethylaminuria; disorders of lipid metabolism including but not limited to: Hyperlipoproteinaemias including Familial hyperchylomicronaemia (Type I), Familial hyper-lipoproteinaemia (familial hypercholesterolaemia) (Type IIA),

Combined hyperlipidaemia Type IIB, Broad – disease (Type III) Pre-lipoproteinaemia (Type IV), Hyperchylomicronaemia with pre-lipoproteinuria (Type V); Hypolipoproteinaemias including but not limited to: A – lipoproteinaemia (acanthocytosis), Familial (primary) hypobetalipoproteinaemia, Familial *a*-lipoprotein deficiency (Tangier disease), Familial lecithin-cholesterol acyltransferase deficiency LCAT); lipid storage diseases including but not limited to: Mucolipidosis Type I (lipomucopolysacchariodosis), Mucolipidosis Type II (I cell disease), Mucolipidosis Type III (pseudo Hurler polydystrophy), GM1 generalised gangliosidosis, GM1 juvenile gangliosidosis, GM2 gangliosidosis with hexosaminidase A deficiency (Tay Sachs disease), GM2 gangliosidosis with hexosaminidase A and B deficiency (Sandhoff disease), Niemann-Pick disease (sphingomyelin lipidosis), Metachromatic leucodystrophy (sulphatide lipidosis), Gaucher's disease (cerebroside lipidosis), Fabry's disease (ceremide trihexoside lipidosis), Acid esterase deficiency (Wolman's disease); disorders of calcium, phosphorus, magnesium and other minerals including but not limited to: Hypercalcaemic states including: Thyrocalcitonin deficiency, Vitamin D-resistant (hyperphosphaturic) rickets; Vitamin D-dependent rickets; Deficiency or excess of other minerals such as Copper, for example Wilson's disease; Menkes steely hair syndrome; Iron, for example, Atransferrinaemia, Haemochromatosis, Congenitall iron overload, Zinc, for example, Acrodermatitis enteropathica; and the like.

As with many diseases of other organs, liver transplantation is often a preferred therapy in diseases associated with errors of liver metabolism. For example, liver transplantation is the established therapy for end-stage liver disease. However, as with most other transplant therapies, liver transplantation is limited by the scarcity of suitable donor organs.

The transplantation of hepatocytes has been proposed as an alternative to whole organ transplantation for liver disease (Asonuma, *et al.*, J. Ped. Surg., 27:298-301 (1992)). The authors report that single metabolic deficiencies may be cured with replacement of 12% of liver mass, suggesting a single liver could be utilized for several patients, or partial resection of a living donor's liver could provide the necessary liver mass to treat another person.

The ability of transplanted cells to manufacture and secrete substances of therapeutic value, or provide needed metabolic functions, and so potentially provide an alternative to whole organ transplantation, has led to the development of implantable devices for maintaining cells within an individual in need of treatment.

In order to replace or augment liver function utilizing liver cell transplantation, regardless of the means of cell delivery, it is critical to ensure the survival and growth of the transplanted cells. Previous studies on hepatocyte transplantation have reported that performing a portal caval shunt (PCS) in conjunction with hepatocyte transplantation improves hepatocyte engraftment (Uyama, *et al.,* Transplantation 55:932-935 (1993)). However, patients in need of liver function replacement, such as hemophiliacs or patients in liver failure, are already in a compromised situation, and the burden of a PCS may not be feasible for this population.

The invention disclosed herein provides methods for enhancing the survival and/or maintenance of function of liver cells capable of secreting one or more factors and/or providing one or more metabolic and/or physiologic functions when implanted into a recipient.

The invention disclosed herein in respect of liver cells, including secretory liver cells, for example, human hepatocytes, relates in a preferred embodiment to the preparation and use of a composition of or a device incorporating neonatal liver cells, including, for example, an "association" of or an "aggregate" of neonatal liver cells, such as neonatal hepatocytes. In other preferred embodiments, said composition, association, or aggregate of liver cells may comprise one or more companion cells capable of providing a growth and/or trophic and/or mitogenic function to said liver cells. An association of at least one liver cell with one or more other liver cells and/or companion cells may be mediated directly, for example by cell-to-cell contact, or indirectly, such as, for example, by secreted factors including hormones, cytokines, or growth and/or trophic factors. Such association may be promoted and/or enhanced by the culturing of said one or more liver cells in the presence of allogeneic serum, and may conveniently be achieved by the culturing of

said one or more liver cells in the present of allogeneic serum together with one or more other liver cells and/or companion cells.

We have found that for the preparation of an association or aggregate comprising liver cells and companion cells, co-culturing allows the cells time to grow in the presence of growth factors produced by the companion cells *in vitro* before the transplant. For example, we have found that secretory liver cell function and/or phenotype may be better maintained if they are cultured and/or associated and/or aggregated with companion cells.

In some embodiments, the liver cells and, when present, the companion cells are preferably derived from the same species as the recipient. In other embodiments, either or both of the liver cells and, when present, the companion cells, are from species other than that of the recipient.

We have also found that for the preparation of an aggregate comprising liver cells, for example, neonatal hepatocytes, culturing in the presence of allogeneic serum allows the liver cells time to grow in the presence of growth factors present in the allogeneic serum *in vitro* before the transplant.

By "aggregate" as used herein we mean a grouping of one or more liver cells as referred to herein which, when implanted in to a recipient, are immunologically, but preferably not physiologically, isolated and/or privileged. An aggregate may comprise more than one type of liver cell. An aggregate may also comprise one or more liver cells together with one or more companion cells.

By "companion cells" as used herein we mean one or more cells capable of enhancing survival and/or viability and/or biological function of the liver cells with which they are cultured and/or associated and/or aggregated. For example, the companion cells may provide growth and/or trophic and/or mitogenic factors to said liver cells, or may provide one or more growth and/or trophic and/or mitogenic functions to said liver cells.

By "liver cells" we mean cells from the liver or associated organs, including the gall bladder, bile duct and hepatic vessels, including, for example hepatocytes, non-parenchymal liver cells, gall bladder cells including gall bladder epithelial cells and gall bladder endothelial cells, liver vessel endothelial and epithelial cells, and

cells, including genetically modified cells, capable of performing metabolic or physiologic functions normally performed by the liver and/or associated organs and/or expressing and/or producing and/or secreting a biologically active molecule. Such a biologically active molecule is also referred to herein as a factor. The biologically active molecule may be selected from but not limited to one or more of the following; blood clotting factors (for example, FVIII, FIX, von willebrand factor), growth and/or differentiation factors (for example, growth hormone and analogues thereof, insulin-like growth factor and analogues thereof, hepatocyte growth factor and analogues thereof, fibroblast growth factor and analogues thereof), and enzymes (such as glutaryl coenzyme A, the deficiency of which causes type 1 glutaric aciduria).

In preferred embodiments, said liver cells are neonatal liver cells.

By "neonatal" we mean of, or derived from, new-born and/or recently born mammals and/or designating or relating to the period just after birth, wherein said period varies from species to species. For instance, in humans the neonatal period is considered to be the first four weeks following birth, and for example, in pigs is considered to be the first seven to ten days following birth.

By "secretory liver cells" we mean cells from the liver or associated organs, including the gall bladder, bile duct and hepatic vessels, capable of secreting one or more factors normally secreted by the liver or associated organs.

By "allogeneic serum" we mean serum suitable for cell culture derived from the same species as that from which the cells were derived. In examples of the present invention where secretory cells and companion cells from different species are co-cultured, an allogeneic serum is one derived from the same species as that from which the secretory cells were derived.

By "factor" we mean a biologically active molecule produced by a cell.

By "long term" we mean a period of more than a week, typically extended to 2 -6 weeks or more.

By "extended period" we mean a period of more than a week, preferably more than two, three, four, five or six weeks or more.

Transplantation of neonatal liver cells, optionally with companion cells, has been investigated as a means of achieving long-term maintenance of liver cell phenotype and/or function, such as the capability to produce and/or secrete one or more factors and/or provide one or more metabolic and/or physiologic functions. The methods, compositions, devices, associations and aggregates of the present invention enable neonatal liver cells to survive, grow, and/or proliferate when transplanted into a recipient.

Our studies described herein show that liver cells, and in particular neonatal liver cells, can, when isolated using the methods herein described, maintain a liver cell phenotype and/or function (including secretion of one or more factors and/or liver cell metabolic and/or physiologic function), survive, and indeed proliferate when cultured according to the methods herein disclosed. Furthermore, the studies presented herein show that neonatal liver cells, when isolated and cultured using the methods described herein maintain liver cell phenotype and/or function in the long-term transplantation into a recipient.

We believe without wishing to be bound by any theory that the isolation and/or culturing of said liver cells utilising allogeneic serum is at least in part responsible for the maintenance of liver cell phenotype and/or function observed.

Cotransplantation of liver cells with companion cells has been investigated as a means of achieving:

(a) protection against immune rejection; and

(b) stimulation of survival, growth, and the mitotic rate of liver cells so that they release physiologically appropriate and/or effective amounts of one or more factors, and/or maintain the capability to provide one or more metabolic and/or physiologic functions, survive longer, and/or proliferate when transplanted into a recipient.

We believe without wishing to be bound by any theory, that the provision of growth and/or trophic and/or differentiation factors and/or functions by said companion cells is at least in part responsible for the maintenance of liver cell phenotype and/or function, growth, survival, and/or protection from immune rejection of transplanted liver cells. As an example of companion cells useful in the

present invention, Sertoli cells are known to play a critical role in various physiological activities such as the synthesis of certain growth factors, for example, insulin-like growth factors 1 and 2 (IGF-1, IGF-2, respectively) and epidermal growth factor (EGF), immunomodulation, possibly as a result of increased secretion of transforming growth factor-beta 1 (TGF-β1), and an anti-apoptotic (cell death inhibitory) function.

Our invention relates in part to the cotransplantation of companion cells with liver cells, and in particular secretory liver cells, such as hepatocytes, as associations and/or aggregates such that the companion cells can act as "nursing" cell systems for the liver cells, providing both efficient immunoprotection and enhancement of their functional performance and longevity.

This approach is complementary to, and synergistic with, other approaches for providing immunoprotection and functional longevity for transplanted liver cells.

In particular our invention deals with the use of liver cells, including neonatal liver cells, in associations, aggregates and compositions in:

- ***Alginate-encapsulated form*** – to provide additional immune protection of the transplanted liver cells. The feasibility of microencapsulating neonatal liver cells and transplanting same is demonstrated in Example 7 herein, and that of co-microencapsulating Sertoli cells with liver cells is demonstrated in Example 6 herein. The efficiency, safety, and function of transplants of such alginate encapsulated liver cell compositions and aggregates in experimental animals.
- ***Subcutaneous implant devices*** that allow the development of a prevascularised allogeneic collagen reservoir for the placement of the liver cell aggregates or compositions. Preferably, the implant device is cell impermeable but protein or secreted factor permeable, such as the "TheraCyte" device available from TheraCyte, Inc., Irvine, Calfornia.
- ***Matrix preparations*** – in which liver cell aggregates or compositions are cultured on gelatin, collagen and/or other matrices supplemented with natural carbohydrate polymers.

- ***Plasma Thrombin Clot*** – Allogeneic plasma clots produced with allogeneic thrombin as a biocompatible containment device.

The maintenance of secretory liver cell function and enhancement of secretory liver cell survival achieved through the co-culturing of secretory liver cells and companion cells is demonstrated herein. For instance, Example 1 herein discloses the effect of co-culturing hepatocytes with growth arrested and non-arrested fibroblasts on the maintenance of a secretory liver cell phenotype.

The invention further relates in part to culturing liver cells, and in particular secretory liver cells, such as hepatocytes, in the presence of allogeneic serum and/or companion cell-derived factors. The Applicants believe, without wishing to be bound, that through the provision of growth and/or trophic and/or mitogenic factors, allogeneic serum provides an enhancement of liver cell function and/or a maintenance of liver cell phenotype and/or longevity *in vitro*, which may persist *in vivo*. Similarly, the Applicants believe without wishing to be bound by any theory, that companion cell-derived factors are able to provide an enhancement of liver cell function and/or a maintenance of liver cell phenotype and/or longevity *in vitro*, which may persist *in vivo*.

The effects of culturing liver cells in media supplemented with allogeneic serum on the maintenance of liver cell function and enhancement of liver cell survival is demonstrated herein. For instance, Example 1 herein discloses the effect of culturing pig hepatocytes in media supplemented with porcine serum on the maintenance of a Factor VIII secretory cell phenotype.

Similarly, the effect of culturing secretory liver cells in media supplemented with companion cell-derived factors on the maintenance of liver cell function and enhancement of liver cell survival is demonstrated herein. For instance, Example 1 herein discloses the effect of culturing pig hepatocytes in media supplemented with fibroblast-derived growth media on the maintenance of a Factor VIII-secretory liver cell phenotype.

The following Examples are provided to illustrate but not to limit the invention in any manner.

EXAMPLE 1: Isolation of liver and gall bladder cells and optimisation of culture conditions

1.1 Isolation of hepatocytes

Hepatocytes were isolated from neonatal porcine liver using our standard procedure as follows. Following surgical removal, the donor livers were transferred to a clean room facility for further processing in a cold plastic container in 50ml tubes containing cold Hank's Balanced Salt Solution (HBSS) with 0.2% human serum albumin (HSA) added. The liver cells were isolated by digestion of the minced liver via a major modification of the standard (Ricordi's) collagenase digestion procedure. Using aseptic technique, the liver was trimmed of excess fat, blood vessels and connective tissue, minced, and digested with Liberase® (0.2 mg/ml) in a shaking water bath (120 rpm) for 10 minutes. The digestion step was repeated twice. The digestion was performed using lignocaine mixed with the Liberase® solution to avoid cell damage during digestion. Following the digestion process, the cells were passed through a sterile 400mm mesh into a sterile beaker. Following the isolation, liver cells were placed into tissue culture in various media as described herein.

1.2 Optimization of culture conditions

To optimise the culture conditions for survival of liver cells and the maintenance of a liver cell phenotype, different media supplemented with various additives were assessed.

Culture Media: Hepatocytes were grown in different liquid media on a number of different surfaces and on surfaces coated with different matrices. Optimum growth was found with the medium DMEM/F 12 (1:1 by volume; Invitrogen Corporation, USA) supplemented with 0.5 U/ml insulin (Novo Nordisk, Denmark), 7 ng/ml glucagon (Novo Nordisk, Denmark), 7.5 μg/ml hydrocortisone (Pharmacia, USA), 1 ml/0.5L Cyproxin 200 (Bayer, Germany)) plus, for example, the additives cyproxin, nicotinamide (10mmol/L), and allogeneic serum (10% by volume). The best surface matrix for hepatocyte growth was found to be collagen.

1.3 Liver cell environment

The effect of the environment in which the liver cells were cultured on liver cell growth and function *in vitro* was assessed as follows.

Cell-extracellular matrix interaction: The effect of interactions between the liver cells and an extracellular matrix on cell viability and maintenance of cell-specific function *in vitro* was assessed using collagen as exemplary extracellular matrix.

Hepatocytes were isolated following our standard procedure. Aliquots of hepatocytes were put into a collagen-coated flask and a non-coated flask. DMEM growth media (GM) supplemented with insulin 0.5U /mL, glucagons (7ng/ mL), hydrocortisone (7.5ug/mL), and 10% (by volume) porcine serum (PS; Invitrogen Corporation, USA) was used. The effect of allogeneic serum supplementation on cell viability and function in the presence of a liver cell-extracellular matrix interaction was assessed, using as a control hepatocytes grown in a collegan-coated flask in growth media lacking procine serum.

Photomicrographs of the cell cultures were taken after 15 days and 18 days in culture.

At both 15 and 18 days of culturing, hepatocytes grown in the presence of a collagen extracellular matrix formed a confluent monolayer irrespective of the presence or absence allogeneic serum in the growth medium. In contrast, hepatocytes grown in the absence of a collagen extracellular matrix formed multiple foci. The functional significance of the different *in vitro* morphologies observed was evaluated with a test on hepatocyte function. Growth media was collected from each flask every fourth day for analysis of albumin production to check the function of hepatocytes during culture. Results for albumin production are presented in Example 2 herein.

Cell-cell interaction: Human fibroblasts were used as companion cells to study the role of cell-cell interaction in fibroblast-hepatocyte co-cultures. Growth-arrested fibroblasts and non-arrested fibroblasts were co-cultured with hepatocytes under the following experimental conditions: 700 000 non-arrested fibroblasts : 250 000 hepatocytes in GM supplemented with 10% fetal bovine serum (FBS) or 10%

PS; a confluent monolayer of fibroblasts arrested with mitomycin C : 250 000 hepatocytes in GM supplemented with 10% FBS or 10% PS.

Cell morphology was assessed by photomicrography after 10, 16, 30, and 37 days in co-culture. For each cell preparation, a flask in which the cells were cultured in serum-free GM was used as a control. The effect of a 3-dimensional support structure on the cell-cell interactions was also investigated, using hepatocytes grown on nylon mesh coated with mitomycin-arrested human fibroblasts. Briefly, nylon mesh was successfully coated with human fibroblasts. The fibroblasts were then arrested with mitomycin. Hepatocytes were placed in the flask with the mesh. After 5 days the mesh was washed with fresh medium and put into a new flask.

Non-arrested fibroblasts quickly overgrew hepatocytes. Cells formed a confluent monolayer even in GM without sera. The control flask with hepatocytes grown in GM without sera was empty after 7 days in culture (data not shown), indicating these conditions could not support hepatocyte survival.

Hepatocytes grown with arrested fibroblasts in GM supplemented with 10% PS presented better morphology compared to cells in GM supplemented with 10% FBS. Cell viability as assessed by morphology was optimal after culturing for 2-3 weeks.

Optimisation of growth media: The effect of supplementation of growth media with various sera on hepatocyte growth and fuction was assessed. Hepatocytes (250 000 cells per flask) were grown in collagen coated flasks under the following experimental conditions: GM supplemented with 5% porcine serum (PS) and 5% Sertoli-conditioned growth media prepared as described below, GM with 5% PS and 5% pig skin fibroblast-conditioned growth media prepared as described below, and 10% PS.

Sertoli-conditioned growth media was prepared as follows: Sertoli cells were cultured for at least 24 hours before growth media was collected and filtered through an 8 micron filter to remove cells. The filtered media was then diluted in a 1:1 ratio with DMEM before use.

Pig skin fibroblast-conditioned growth media was prepared as described for Sertoli-conditioned growth media above, with the substitution of fibroblasts for Sertoli cells. The pig skin fibroblasts were isolated as follows: Pig skin was soaked in DMEM plus cyproxin, and fungizone, for 20 minutes, then cut with a scalpel into small pieces. Pieces of tissue were then placed in a standard culture flask with DMEM media supplemented with 10% PS. After one week of culture, pieces of tissue were removed, the remaining cells adhering to the flash were washed, and fresh growth medium was added.

Supplementation of growth media with 10% PS, or 5% PS and 5% pig skin fibroblast-conditioned growth media, yielded better hepatocyte viability than supplementation of growth media with 5% PS and 5% Sertoli-conditioned growth media. Cell viability as assessed by morphology was optimal after culturing for two to three weeks.

Analysis of the morphology of the cells in culture showed that for hepatocyte viability and growth *in vitro*, supplementation of growth media with allogeneic (porcine) serum was preferable to supplementation with foetal bovine serum. Furthermore, supplementation of growth media with either 10% PS, or 5% PS and 5% fibroblast-conditioned growth media was preferable to supplementation with 5% porcine secrum and 5% Sertoli-conditioned media.

1.4 Isolation of non parenchymal liver cells and gall bladder cells

Non-parenchymal liver cells (NPC) were isolated following the procedure by Gerlach *et al.* (2001), with the following modifications. Briefly, the liver was cut into small pieces, and washed three times to remove erythrocytes. Tissue was then digested with Liberase® (0.2mg/ml) for 30 min. Digestion was stopped with 10% porcine serum. Hepatocytes were sedimented at 50 g for 5 min. Non-parenchymal cells were sedimented at 600 g for 10 min, and then washed three times in PBS. Cells were counted, and their viability was checked by trypan blue exclusion as described above. Cells were plated at 10,000 cells/flask. At day 7 in culture, the cell count and viability check was repeated. Supernatant was collected for albumin ELISA and FVIII functional tests.

10,000 NPC were isolated from the same neonatal pig liver. Viability of cells immediately after isolation was 100%. Maximal rate of FVIII coagulation was 0.2% at day 5 in culture.

Epithelial and endothelial cells were isolated from pig gall bladder and liver vessels. Briefly, gall bladder was thoroughly washed with sterile DMEM to remove bile, cut into pieces, and digested with Liberase® (0.2 mg/ml) for 30 min. Cells were then washed with DMEM three times. Cell count and viability tests were performed immediately after isolation, and again at day 7, day 16, and day 28 in culture. Cells were plated in 25cm ^{2} flasks at 15 x 10^6 cells/flask. Albumin and FVIII release functional tests were performed at day 7 in culture.

31 x 10^6 cells were isolated from pig gall bladder and liver vessels. Viability immediately after isolation was 100%. After 16 days in culture, cell survival rate was 120%. Maximal albumin production was 2.27 ug/ml/4h, and the maximal rate of FVIII coagulation was 3.7%.

EXAMPLE 2: Characterization of cells: secretory function and cell markers

To determine whether liver cells in culture maintain liver cell function, for example, the ability to produce or secrete liver cell secreted factors, the following experiments were conducted.

Albumin secretion

Albumin is the major plasma protein secreted by hepatocytes. In conventional culture methods, the rate of secretion of albumin drops rapidly during culture. Hepatocyte albumin secretion was used herein as a test for the maintenance of normal hepatocyte function, and thus the maintenance of a liver cell phenotype, for example, in culture.

Factor VIII (FVIII)

The liver and the reticularendothelial system are thought to be primary sites of FVIII production. Liver transplant corrects FVIII deficiency in persons with hemophilia. FVIII is secreted as a glycoprotein into the circulation as a heterodimer. FVIII production may be used to characterise liver cells. FVIII present in the

filtered supernatant was measured using the Dade-Behring clotting system (Coatest VIII;c/4 from Chromogenix).

2.1 Albumin and FVIII production by liver cells

Hepatocytes isolated from neonatal porcine liver according to our standard protocol were cultured in the preferred liquid medium for up to 5 weeks on a matrix of collagen as described above. The production of FVIII and albumin was then determined as follows.

250,000 hepatocytes per flask were cultured in medium supplemented with additives. Supernatant from the cultured cells was removed and discarded, and cultures were washed twice with PBS. 5 ml serum-free media was then added to the culture flasks. A 1 ml aliquot of media was immediately removed from the flask and used for a baseline measurement. Cells where then incubated at 37°C for 4 hours. After incubation, supernatant was collected, and filtered to remove cellular debris. Albumin present in the filtered supernatant was measured using the pig albumin ELISA Core Kit (Komabiotech) following the manufacturer's protocol. FVIII present in the filtered supernatant was measured using the Dade-Behring clotting system.

Production of FVIII was observed, with cells producing considerable quantities after 2 weeks in culture and maintaining the output of FVIII for 5 weeks (see Figure 1) at which time the experiment was terminated.

The output of 250,000 hepatocytes over a 4-hour period gave a FVIII value approximately 8% of normal blood levels. Since the half life of FVIII in human blood is 36 hours, this rate of production is very substantial. It should also be noted that the production of albumin by these hepatocyte preparations correlates well with FVIII production. Albumin is a typical liver product, and production of albumin indicates the hepatocytes were healthy.

These results indicate that neonatal hepatocytes are able to maintain a secretory liver cell phenotype during prolonged cell culturing.

In another experiment, media harvested from hepatocytes grown under the various conditions described in Example 1 above, (and reproduced below) were analysed for albumin content as a determinant of hepatocyte function.

1- Hepatocytes on mitomycin arrested fibroblasts in growth media supplemented with 10% FBS;
2- Hepatocytes on mitomycin arrested fibroblasts in growth media supplemented with 10%PS;
3- Hepatocytes on mitomycin arrested fibroblasts in growth media without serum, see;
4- Hepatocytes on collagen coat in growth media supplemented with 10% PS;
5- Hepatocytes on collagen coat in growth media supplemented with 5% PS and 5% fibroblast conditioned growth media;
6- Hepatocytes on collagen coat in growth media supplemented with 5% PS and 5% Sertoli conditioned cells growth media.

Albumin content was analysed as follows: Aliquots of supernatant were taken at day 5, 7, 10, 13, 32 and 54 of culture. First, supernatant from the cultured cells was removed and discarded, and cultures were washed with PBS. 5 ml of growth media without serum was then added. A 1 ml aliquot of this growth media was immediately taken from the flask, and used for a baseline measurement of albumin production. Cells were then incubated at 37°C for four hours. After incubation, supernatant was collected and filtered to remove cellular debris. Albumin present in the filtered supernatant was measured using pig albumin ELISA Core Kit (Komabiotech) according to the manufacturer's protocol. Growth media prepared in accordance with the original culture conditions was then added to the flasks for continued culturing.

As show in Figure 2, the highest albumin production was observed in cultures supplemented with porcine serum. Hepatocytes grown in media supplemented with PS on arrested fibroblasts yielded a maximum albumin release of 19.5 μg/ml for 4 hours at day 10 in culture (see Figure 2). Hepatocytes grown on

collagen matrix in GM supplemented with PS showed a maximum albumin release at day 32 in culture of 38.3 μg/ml for 4 hours.

Maximum albumin production for hepatocytes grown in GM supplemented with PS and with fibroblast-conditioned growth media was 3.76 μg/ml at day 32 of culture. Maximum albumin production of hepatocytes grown in GM supplemented with PS and Sertoli-conditioned growth media was 4.56 μg/ml at day 13 in culture.

These results demonstrate that hepatocyte function, as assessed by albumin production was best maintained when the hepatocytes were grown in collagen coated flasks in growth media supplemented with 10% PS. A liver cell-extracellular matrix interaction and the presence of allogeneic serum are important for the maintenance of a liver cell phenotype in long term tissue culture.

In another experiment, liver cells were isolated from male and female large White/Landrace cross neonatal pigs according to the method described above. Cells were seeded at a density of 2 x 10^6 viable cells in 25 cm collagen-coated flasks (Sigma, USA) and were maintained at 37^0C in a humidified incubator in an atmosphere of 95% *air* and 5% CO_2. Cells were cultured in DMEM/F12 media, and 10% porcine serum. After 48 hours the cells were rinsed with PBS and fresh culture media was added. Fresh media was subsequently added every 2-3 days as needed.

Functional tests to measure albumin release and Factor VIII release were performed at 1, 2, and 3 weeks as described above. Cell numbers and viability was also determined. The viability of the porcine cells was excellent (>90%) at all time points tested using the current methods (see Table 2.1). During the initial 24-48 hours post isolation the numbers of viable cells did decline (data not shown). However, the numbers of viable liver cells increased almost linearly from that point on until the conclusion of the studies 3 weeks later.

The maintenance of liver cell function of the isolated liver cells was confirmed using albumin and Factor VIII release. Both albumin and Factor VIII were detectable 1 week post isolation. Quantitative determinations demonstrated

that, on a per cell basis, the release of both albumin and Factor VIII increased markedly from 1 to 3 weeks in culture (Table 2.1).

Table 2.1: In Vitro Characteristics of Isolated Neonatal Hepatocytes

Time Post Isolation	Cell Viability %	Cell Number 10^6 (proliferation)	Albumin Release (μg/ml)	Factor VIII Release (mU/ml)
1 wk	91.3 (90.0-93.0)	0.25 (0.2-0.3)	1.7 (0-2.8)	1.1 (.03-1.9)
2 wks	89.8 (86.5-93.4)	1.1 (0.9-1.4)	11.6 (8.3-14.8)	3.3 (2.4-3.1)
3 wks	89.2 (84.9-93.4)	2.6 (2-3.25)	29.4 (26.3-32.5)	11.9 (11.3-13.4)

Data for albumin and Factor VIII release are expressed as per 1 million cells.

2.2 Indocyanine Green Uptake

Indocyanine green (ICG) is a non-toxic organic anion that is used in clinical tests to evaluate liver function, as it is eliminated exclusively by hepatocytes *in vivo*. ICG uptake has been used to identify differentiated hepatocytes from stem cells in culture (Yamada et al., 2002). In the present study, cellular uptake of ICG was used to identify hepatocytes in culture in a screening method to identify the best culture conditions for the long term maintenance of hepatocyte function.

ICG was used at a concentration of 1 mg/ml dissolved in 5 ml sterile water and 20 ml DMEM with 10% PS. The ICG solution was added to the cell culture flask and incubated at 37°C for 15 min. After the flask was rinsed three times with PBS, the cellular uptake of ICG was examined by microscopy. After the examination, the flask was refilled with fresh growth media.

Microscopic examination of isolated neonatal hepatocytes showed that approximately 50% of cells were ICG positive.

In another experiment, ICG uptake by neonatal porcine liver cells cultured in media supplemented with 10 % PS in collagen-coated flasks was determined. After 3 weeks in culture, the percentage of ICG positive cells was very high and ranged between 80-90%. After 4 hours of incubation virtually all of the cells released the

ICG that had been taken up, wherein said release is a marker for release after metabolism.

The maintenance of liver cell function in long term tissue culture can be readily assessed by ICG uptake. Furthermore, administration of ICG to liver cells in tissue culture is a useful methodology to manipulate liver cell function and control hepatocyte differentiation *in vitro*.

2.3 Secretory function of different cell types

The importance of the procedures used to isolate liver cells from tissue to the maintenance of liver cell function was assessed, using FVIII secretion and albumin production as markers for secretory liver cell function. The ability of non-parenchymal (NPC) cells from the liver, and epithelial and endothelial cells from gall bladder and liver vessels, to exhibit and maintain a secretory liver cell phenotype in culture was also assessed.

One neonatal (one week old) piglet, and one approximately 6 month old pig were used in the following experiments. The supernatant of cultured cells was harvested as indicated, and albumin release and FVIII functional tests (ELISA, and coagulation test as described herein, respectively) were performed. ICG uptake tests were also performed as indicated.

Hepatocytes were isolated from one neonatal pig liver following our standard procedure described herein, with two rounds of digestion with Liberase® (0.2mg/ml) for 10 min. After isolation, cells were counted and plated in 25 cm^2 flasks, with 4.5 x 10^6 cells/flask. Cells were counted after 5 days in culture, and viability was checked by trypan blue exclusion as described above. Albumin and FVIII release were assessed at day 5 in culture.

The production of Factor VIII was measured using a Factor VIII coagulation assay (Coatest VIII:C/4 from Chromogenix) according to the manufacturer's protocol. Percent values for the rate of Factor VIII coagulation are relative to the rate of Factor VIII coagulation at normal blood levels of Factor VIII.

37 x 10^6 hepatocytes were isolated from one neonatal pig liver as described above. Viability of the cells immediately after isolation was 98%. After 5 days in

culture, cell survival rate averaged 60%. Albumin production after 5 days in culture was 4.45 ug/ml/4h. The maximal rate of FVIII coagulation was 0.2%. Staining with ICG was used to identify the percentage of hepatocytes in cell culture. 43% of cells were ICG positive in normal liver preparation after 4 weeks in culture.

Non-parenchymal cells (NPC) were isolated from the same liver used for hepatocyte isolation. Cell yield was low, as expected from previous reports (Gerlach, 2001). Despite this, NPC so isolated were competent to produce FVIII, and indeed were producing approximately the same amount of FVIII as hepatocytes (approximately 0.2%) with about one-third the amount of cells.

Epithelial and endothelial cells from piglet gall bladder and liver vessels were isolated. These cells showed good growth in culture, proliferated under the culture conditions used, and exhibited the highest rate of FVIII coagulation, at 3.7%.

2.4 Proliferation of hepatocytes and maintenance of function in long term cultures

The effects of cell isolation methodology and culture conditions on cell proliferation and secretory function were assessed.

Cell proliferation: Neonatal hepatocytes were isolated according to our standard method described herein. 250,000 cells were seeded in flasks, and cultured in growth media supplemented with 10% FBS. Proliferation of cells in culture was determined at 1 day in culture, 7 days in culture, 14 days in culture, and 21 days in culture.

The number of viable cells, relative to day 1 of culture, in growth media supplemented with 10%FBS was 20% at day 7, 203% at day 14, and 287% at day 21.

Albumin release: Hepatocytes in growth media supplemented with 10% PS or 10% FBS were cultured, and albumin release was checked as described above at 1, 2, 3, 7, 8, and 9 weeks of culture. Pig fibroblasts in growth media supplemented with 10% PS or 10%FBS were used as a negative control.

Maximum albumin release for hepatocytes cultured in growth media supplemented with 10% porcine serum was observed at week 9 (19.1 μg/ml for 4 hours). A rise in albumin production was observed in the negative control, with a maximum albumin release of 0.67 μg/ml at week 3 (see Figure 3).

The low cell viability at day 7 of culture indicated that the hepatocytes were damaged during the isolation step. However, under these conditions the hepatocytes were able to recover and proliferate.

Albumin production was inhibited for several weeks in culture with albumin release considerably lower than that described elsewhere herein. Again however, under these culture conditions liver cell function recovered after 8-9 weeks in culture.

Hepatocytes isolated and cultured as described can be successfully cultured to maintain hepatocyte function, including liver hepatocyte secretory function, for at least 9 weeks *ex vivo*.

2.5 Immunoperoxidase cell characterization

An immunoperoxidase method to distinguish different liver cell populations in culture was developed. Adult pig and neonatal pig livers were stained with hepatocyte specific antigen and for von Willebrand factor to distinguish endothelial cells and hepatocytes. The following markers were used: hepatocyte antigen and cytokeratin for mature hepatocytes; von Willebrand factor for endothelial cells; vimentin for cells of mesenchymal origin; and Factor VIII to identify cells that are the main producers of the factor.

Freshly isolated liver cells or tissues were formalin fixed, paraffin embedded, and sectioned at 2 μm. Unstained slides were deparaffinized in xylene and hydrated in graded alcohols. Slides were treated with 0.5% H_2O_2 for 5 min to block endogenous peroxidase activity. Sections were stained with primary antibody using the DAKO EnVision System according to the manufacturer's protocol. Sections were incubated for 30 min with primary antibody, followed by 30 min incubation

with peroxidase-labelled polymer, and 5 min incubation with substrate-chromogen. Slides were counterstained with hematoxylin.

The following Table summarises the characteristics of the cell populations.

Table 2.2: Characterization of Cell Type

Cell Type	Hepatocytes	Gall Bladder Epithelial cells	Endothelial cells from liver vessels	NPC from liver
Albumin secretion	Positive	Positive	Positive	Positive
FactorVIII secretion	Positive	Positive	Positive	Positive
ICG Uptake	60–95% Positive	<1% Positive	<1% Positive	<30% Positive
Immunoperoxidase Markers				
Alpha feotoprotein	Positive	Negative	Negative	Negative
Cytokeratin CK 7	Negative	10% Positive	Negative	Negative
Vimentin	Negative	Negative	Positive	Positive

NPC = Non parenchymal liver cells

2.6 Co-culture of secretory cell with companion cells

The applicant has co-cultured various combinations of secretory liver cells and companion cells and studied the effects on FVIII production. The data summarised in Table 2.3 shows that FVIII secretion is markedly increased in co-cultures of hepatocytes with companion cells.

Table 2.3: Effect of Co-culturing on FVIII secretion

Tissue or Cell Associations in Co-culture	FVIII Released μU/ml
Experiment A	
Lung Tissue	<0.1
Lung Tissue + Hepatocytes ($0.5x10^6$cells)	0.35
Gall Bladder Epithelial (10^6 cells)	1.02
Gall Bladder Epithelial(10^6 cells) + Hepatocytes ($0.5x10^6$ cells)	0.48
Endothelial (10^6 cells)	<0.1

Endothelial (10^6 cells) + Hepatocytes ($0.5x10^6$ cells)	0.97
Hepatocytes (10^6 cells)	0.58
Experiment B	
Gall Bladder Epithelial ($0.5x10^6$cells)	0.65
Gall Bladder Epithelial ($0.5x10^6$cells) + Hepatocytes ($0.5x10^6$cells)	1.15
Gall Bladder Epithelial ($0.5x10^6$cells) + Hepatocytes ($1.0x10^6$cells)	0.94
Gall Bladder Epithelial ($0.5x10^6$cells) + Hepatocytes ($1.5x10^6$cells)	1.22
Endothelial (10^6 cells)	0.15
Endothelial (10^6 cells) + Hepatocytes (100,000 cells)	0.68
Hepatocytes ($1x10^6$ cells)	0.64

EXAMPLE 3: Cryopreservation of hepatocytes

Hepatocytes were isolated according to our standard procedure described above, and additionally according to our modified procedure, in which digestion with Liberase® during isolation is performed in media supplemented with PS. The isolated hepatocytes were pooled, and then frozen following the standard procedure described under the following three different conditions: in 10%DMSO in FBS; 10% FBS and 10% DMSO in GM; and 10% DMSO in PS. Cells were stored in liquid nitrogen. After one week and three weeks storage, cells were defrosted and viability and recovery were determined. Results are summarised in Table 3.1.

Cells maintained good viability and recovery after storage in liquid nitrogen for one to two weeks. The best viability and recovery of cryopreserved hepatocytes was observed with hepatocytes isolated according to our modified procedure and frozen in 10% DMSO in PS.

Table 3.1: Hepatocyte cryopreservation

		Cells cultured for 3 days		Cells cultured for 30 days	
	Viability	Viability	Recovery	Viability	Recovery
PS/DMSO Modified isolation	96%	83%	72%	80%	60%
GM/DMSO	96%	60%	43%	76%	30%
FBS/DMSO Modified isolation	96%	81%	64%	ND	ND
PS/DMSO Conventional isolation	57%	61%	45%	0%	0%

Viability is the percentage of living cells. Recovery is the percentage of living cells after thawing. ND=Not Determined.

EXAMPLE 4: Ischaemia during cell isolation

The effect of different modes of ischemia during cell isolation and of the isolation methodology itself on liver cell function and the maintenance of liver cell function was assessed as follows.

4.1 Cold Ischemia

The effect of cold ischemia during cell isolation on liver cell function were assessed as follows. Liver was cut into small pieces, and put in a large volume of cold DMEM for 24 hours storage at 4°C. Hepatocytes and NPC were then isolated following the standard procedure as described herein.

251 x 10^6 hepatocytes were isolated from one pig liver using the cold ischemia method described above. Viability immediately after isolation was 21%, with 52 x 10^6 cells surviving isolation. After 4 weeks in culture survival rate was 18%. Maximal FVIII coagulation rate was 1% after 4 weeks in culture.

444 x 10^6 NPC were isolated from the same liver. Viability immediately after isolation was 6.3%. After isolation using the cold ischemia procedure, 95% of cells in culture were positive for ICG.

4.2 Warm Ischemia

The effect of warm ischemia during cell isolation on liver cell function was assessed as follows. Liver was cut into small pieces, and put into a large volume of DMEM and stored at room temperature for 6 hours and for 12 hours. Hepatocytes and NPC were then isolated following the standard procedure as described herein.

These cells were cultured for approximately 7 weeks. After seven weeks in culture hepatocytes isolated using the warm ischemia method as described above were releasing FVIII into the supernatant. Maximal rate of FVIII coagulation for cells prepared according to the standard procedure was 1.6%. For cells prepared using 6 hours of warm ischemia, maximal rate of FVIII coagulation was 2.7%, and for cells prepared using 12 hours of warm ischemia, maximal rate of FVIII coagulation was 1.2% (despite the fact that the flask with hepatocytes isolated using 12 hours of warm ischemia was almost empty with a very small cell count).

4.3 Cold Ischemia with Abattoir Liver

Three methods of cell isolation were assessed using tissue from a single organ collected from an abattoir. The liver was collected and immediately put in cold Hank's solution supplemented with antibiotics and fungizone. Within one hour of collection, the tissue had been processed following the standard procedure described herein. One aliquot of cells was put on a Percoll gradient, another one on Limphoprep system, while the third aliquot was treated as per routine procedure.

Isolation of liver cells from the abattoir liver gave a very poor yield and quality of cells. The best isolation was achieved with the Limphoprep system. Notwithstanding this, due to the very poor yield of viable cells, functional tests were not conducted.

4.4 Cold Ischemia

In another experiment, excellent results in respect of cell yield and liver cell function were achieved using the cold ischemia method developed by the Applicants and described herein. The number of isolated cells was higher using the cold ischemia method than after isolation using the normal method, with 52 x 10^6

viable cells compared to 37 x 10^6 viable cells using the standard preparation methodology.

After a standard protocol preparation, 43% of cells were positive for ICG, a marker for functional hepatocytes. In comparison, 95% of cells were positive for ICG using the cold ischemia method. The FVIII coagulation test showed that cells isolated using the cold ischemia method produced more FVIII (1%), compared to cells isolated using the standard method (0.2-0.5%).

4.5 Warm Ischemia

Excellent results in regard to hepatocyte secretory function were achieved using the warm ischemia isolation method described herein. FVIII coagulation showed that hepatocytes cultured for approximately 7 weeks following isolation using 6 hours of warm ischemia yielded 2.7% of normal FVIII function, compared to 1.6% for hepatocytes isolated using the standard protocol. Hepatocytes cultured for 7 weeks after isolation using 12 hours of warm ischemia showed 1.2% of normal FVIII function. The Applicants believe, without wishing to be bound by any theory, that ischemia during isolation may result in a more robust and virile hepatocyte population in which hepatocyte secretory liver cell function is maintained in long-term culture.

4.6 Cold Ischemia

In a further experiment, the effects of ischemia during isolation and culture conditions on liver cell function of isolated pig hepatocytes were assessed. One week old Auckland Island (AI SPF3) piglets were used for hepatocyte isolation. Hepatocytes were isolated from one neonatal pig liver according to our standard procedure described herein, with two rounds of digestion with Liberase® (0.2mg/ml) for 10 min. After isolation, cells were counted and plated in 25 cm^2 flasks at 4.5 x 10^6 cells/flask.

Hepatocytes were also isolated using the cold ischemia method described herein. Briefly, liver was cut into small pieces and put in a large volume of cold

DMEM with 10% allogeneic (porcine serum) and stored for 24 hours at 4°C. Hepatocytes were isolated following the standard procedure described above.

Viability of the cells immediately after isolation using the standard procedure was 97%, and 86% after isolation using the cold ischemia method. Cells were seeded at 2 x 10^6cells per flask. Cell count dropped dramatically at day 2 in culture then increased to exceed the original level after two weeks in culture (see Figure 4).

Tests on albumin and FVIII release were performed on the supernatant of cultured cells as described above. Hepatocytes isolated using the cold ischemia method showed higher albumin release than hepatocytes isolated according to the standard protocol at each time point (see Figure 5).

For both hepatocytes prepared according to the standard protocol and hepatocytes isolated using the cold ischemia method, maximum FVIII secretion was observed after 3 weeks in culture (see Figure 6). Maximal rate of FVIII coagulation was 0.12 units/ml/10^6 cells (or 5.3% per 1.6 x 10^6 cells).

4.7 Isolation of Epithelial and endothelial cells

Epithelial and endothelial cells were isolated from pig gall bladder and liver vessels as follows. Briefly, gall bladder was thoroughly washed with sterile DMEM to remove bile, cut in pieces, and digested for 30 min Liberase® solution (with 0.2 mg/ml). Cells were then washed with DMEM three times. Cell count and viability tests were performed immediately after isolation, and at day 7, day 16, and day 28 in culture. Cells were plated at 15 x 10^6/flask in 25cm ^{2} flasks.

Considerable FVIII release by epithelial and endothelial cells isolated from pig gall bladder and liver vessels was observed after 4 weeks in culture (see Figure 6).

The gall bladder cell preparation also showed considerable amount of FVIII release after 4 weeks in culture.

In summary, the standard isolation technique using low Liberase® concentration provides good results with respect to cell yield and viability. In this instance, the viability of cells isolated using the Applicant's cold ischemia protocol (86%) was comparable to that achieved using the standard method. However,

hepatocytes cultured following cold ischemia isolation showed better functional recovery compared to those isolated by the standard procedure, as demonstrated by higher albumin release.

Irrespective of the isolation method used, the cultured cells maintained liver cell function in culture, with maximum FVIII secretion usually observed at 3 weeks in culture for hepatocytes. The applicant's data shows that epithelial and endothelial cells isolated from pig gall bladder and liver vessels secrete FVIII in culture, in excess of that observed at the same timepoint with hepatocytes.

EXAMPLE 5: Encapsulation of hepatocytes

The effects of cell isolation methodology and culture conditions on cell viability and maintenance of liver cell function were assessed using mouse hepatocytes. Additionally, methods to encapsulate mouse hepatocytes as single cells and as cell clusters were developed.

Six adult CD1 mice, one month of age, were used for isolation of adult mouse hepatocytes. Isolation of neonatal mouse hepatocytes was performed using 12 5-7 day old CD1 mice and repeated with 45 5-7 day old CD1 mice. The average weight of the isolated neonatal livers was approximately 0.07 grams.

Hepatocytes were isolated from mouse liver following the standard procedure described herein, with three rounds of digestion for 10 min using Liberase® at a concentration of 0.2mg/ml. After isolation, cells were counted and plated in 25 cm^2 flasks.

Cells were isolated using the cold ischemia method as follows. Liver was cut into small pieces, and put in a large volume of cold DMEM and stored for 24 hours at 4°C. Hepatocytes were then isolated following the standard procedure described herein using Liberase® (0.2 mg/ml).

Viability of hepatocytes after isolation from adult mouse liver was 49%. Viability of adult mouse hepatocytes after isolation using the cold ischemia protocol was 2%. Cell count visibly dropped during the 1-2 weeks in culture. ICG showed positive staining for hepatocytes. Cells did not survive longer than 2 weeks in culture.

Viability of cells immediately after isolation from neonatal mouse liver was 31% and 23% for the first and second preparations, respectively. Cells were cultured in routine media supplemented with 10% FBS. After one week in culture hepatocytes were encapsulated using 1.5% alginate for the formation of capsules.

Single cells and cell clusters were encapsulated, and the integrity of capsules was verified by microscopy. For both single cells and cell clusters, there were no cells visibly embedded in the capsule wall, and capsules were of even shape and size, approximately (200 μm in diameter).

The standard isolation technique, with low Liberase® concentration (0.2 mg/ml), yielded lower cell viability with mouse hepatocytes than that observed with pig hepatocytes.

Although viability of neonatal hepatocytes after isolation was lower than that of adult hepatocytes (31% and 23% compared to 49%), neonatal cells showed much better survival during culture.

Encapsulation mouse hepatocytes in alginate (1.5%) resulted in capsules of good shape and integrity, with no cells embedded in the capsule walls, indicating that liver cells from mammals other than pigs are amenable to aggregate formation and/or encapsulation.

EXAMPLE 6: Transplantation

Methods to prepare compositions, associations, aggregates, and devices for the transplantation of liver cells were developed, and their suitability for transplantation was investigated.

Two modes of hepatocyte transplant delivery were considered in the following experiment: alginate encapsulation, and incorporation into the TheraCyte device. Cell viability, both *in vitro,* and *in vivo* and the ability of the cells to maintain secretory liver cell function and to release Factor VIII from the alginate capsule or TheraCyte device was assessed.

Alginate Encapsulation

Hepatocytes were encapsulated in alginate using different alginate concentrations (1.5%, 1.6%, and 1.7%) to assess the effect of alginate concentration

on Factor VIII release from the capsules. Encapsulation using polyornithine (PLO) versus polylysine (PLL) coating was performed to assess capsule integrity and Factor VIII release. Additionally, both single cells and cell clusters were encapsulated.

Capsules with single hepatocytes and cell clusters were transplanted into three CD1 mice. After three weeks the capsules were retrieved. Cell viability was more than 85% by trypan blue and acridine orange/propidium iodate staining. Approximately 65% of the retrieved cells were ICG positive indicating that the majority of retrieved cells were mature hepatocytes.

Incorporation into TheraCyte Device

One million hepatocytes were loaded into two TheraCyte devices. One TheraCyte device was maintained *in vitro* in culture media supplemented as described above with 10% allogeneic serum. Albumin and Factor VIII were released from the TheraCyte maintained *in vitro* (see Figures 7 and 8, respectively). Factor VIII coagulation essays indicated that Factor VIII activity released from the TheraCyte device was approximately 65% of that released by an equivalent amount of cells when free in culture.

The second TheraCyte device was transplanted subcutaneously in a CD1 mouse. The TheraCyte was retrieved after three weeks. Cell viability was 98%, and ICG staining indicated more than 80% of the cells were mature hepatocytes. Importantly, histological studies indicated there was no evidence of an inflammatory reaction in the tissues surrounding the transplantation site.

In another experiment, the survival and maintenance of liver cell function of neonatal porcine liver cells following transplantation into a xenogeneic host was determined. Neonatal porcine liver cells were cultured in media supplemented with 10 % PS in collagen-coated flasks. Cells were removed from the flasks using Protease (TrypLE Select, Gibco, USA) and were briefly rinsed in 3 ml of PBS. Cells were then suspended at a concentration of 1 x 10^6/20 µl and loaded into 20 µl immunoisolatory 20 µl Theracyte devices using standard procedures as detailed by the manufacturer. CD1 mice (N=3) were anesthetized using Halothane (2%) and a

small 1 cm incision was made in the abdomen. The device was carefully placed overlying the liver and the incision was sutured closed. All procedures were performed using sterile techniques. Eight weeks later, the devices were removed from the animals for determinations of cell viability. One device was removed and immediately fixed in 10% buffered formalin. The device was embedded in paraffin and 3 μm thick microtome sections were stained for hematoxylin and eosin (H&E). The remaining 2 devices were removed and cut open to flush the encapsulated cells from the device for viability determinations and ICG analysis.

The Theracyte devices were well-tolerated in all of the transplanted animals. All three mice remained extremely healthy and vigorous during the 8 week transplant period. Post-mortem analysis did not reveal any signs of inflammation as the peritoneal surfaces appeared normal, pale and glistening. As anticipated, the devices were slightly adhered to the surrounding organs including the liver but were removed with little disturbance of the surrounding tissue/vasculature.

H&E-stained sections of a retrieved device at 8 weeks supported the biocompatibility of the devices after 8 weeks in the intraperitoneal cavity. The tissue adherent to the Theracyte devices involved organised fibrous tissue. There was notable absence of any acute inflammatory cell reaction, and absence of pronounced round cell infiltrate. Within the device there were numerous, well defined clumps of viable liver cells randomly distributed throughout the lumen. The liver cells were removed from the remaining 2 cell-loaded devices and tested for viability and function using the ICG test described above. There was no diminishment in the numbers of viable/functional cells relative to pre-transplant values as the proportion of ICG-positive cells was 80-90% after 8 weeks in vivo. Further analysis using trypan blue exclusion confirmed that approximately 90% of the retrieved cells were viable.

The above studies show that liver cell viability, function, and phenotype can be maintained for extended periods in transplanted alginate capsules and transplantation devices such as the TheraCyte device. Additionally, the

transplanted liver cells do not provoke an immune reaction and are immunologically isolated from the recipient.

All patents, publications, scientific articles, and other documents and materials referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such referenced document and material is hereby incorporated by reference to the same extent as if it had been incorporated by reference in its entirety individually or set forth herein in its entirety. Applicants reserve the right to physically incorporate into this specification any and all materials and information from any such patents, publications, scientific articles, web sites, electronically available information, and other referenced materials or documents.

The specific methods and compositions described herein are representative of various embodiments or preferred embodiments and are exemplary only and not intended as limitations on the scope of the invention. Other objects, aspects, examples and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. Thus, for example, in each instance herein, in embodiments or examples of the present invention, any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms in the specification. Also, the terms "comprising", "including", containing", *etc.* are to be read expansively and without limitation. The methods and processes illustratively described herein suitably may be practiced in differing orders of steps, and that they are not necessarily restricted to the orders of steps indicated herein or in the claims. It is also that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a host cell"

includes a plurality (for example, a culture or population) of such host cells, and so forth. Under no circumstances may the patent be interpreted to be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

DATED THIS 3rd DAY OF September 2004
AJ PARK
PER Howdale
AGENTS FOR THE APPLICANT

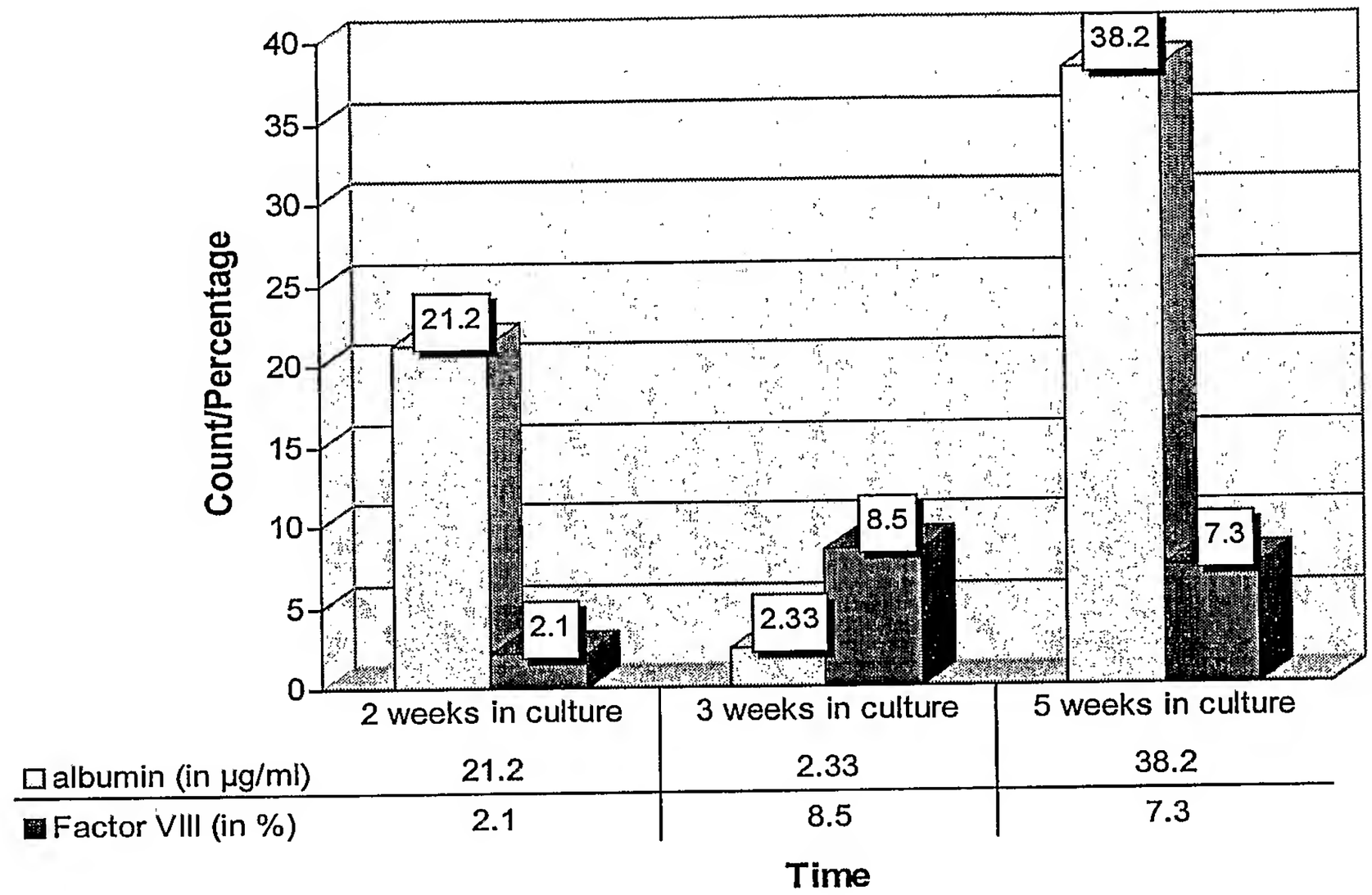
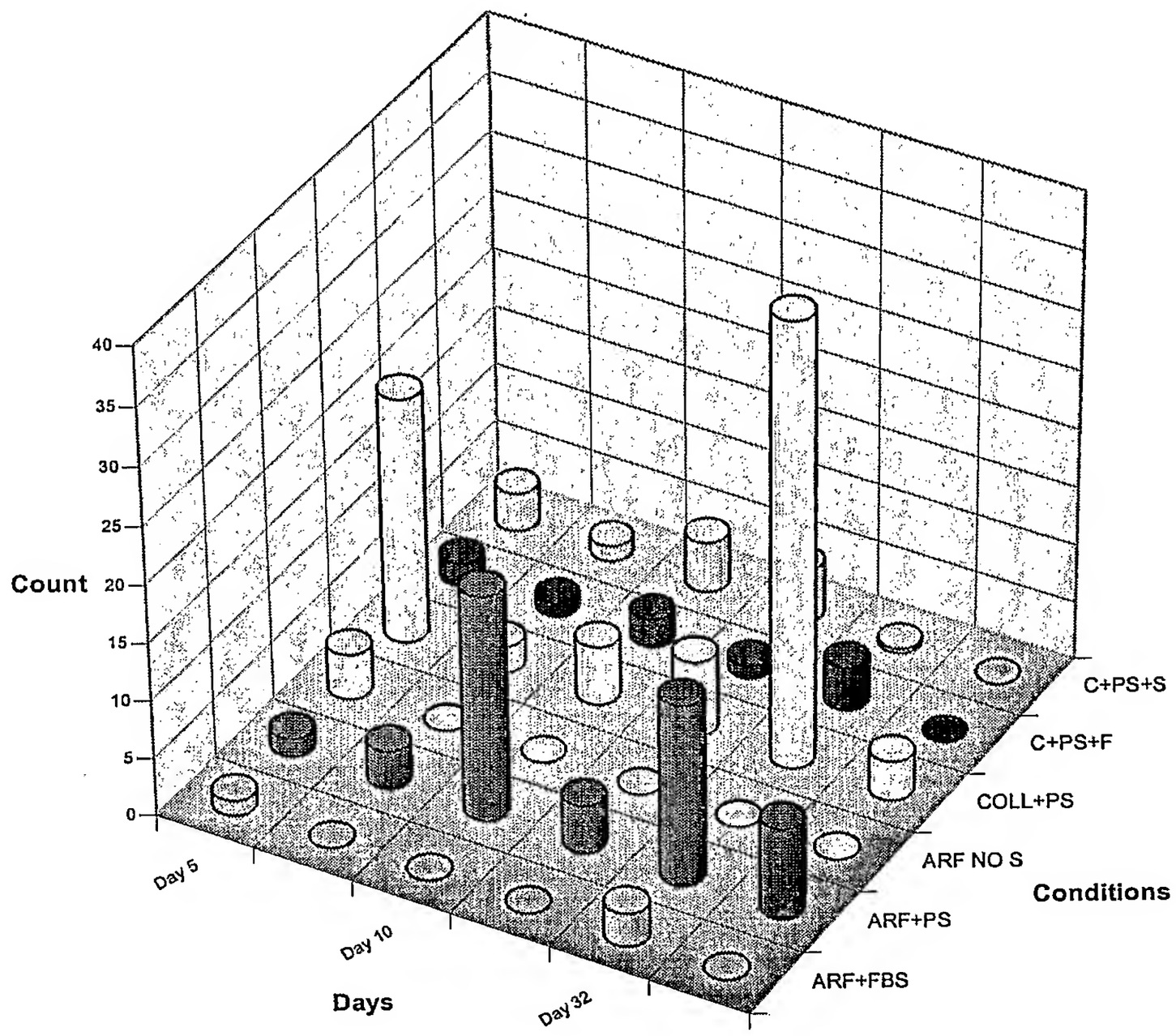
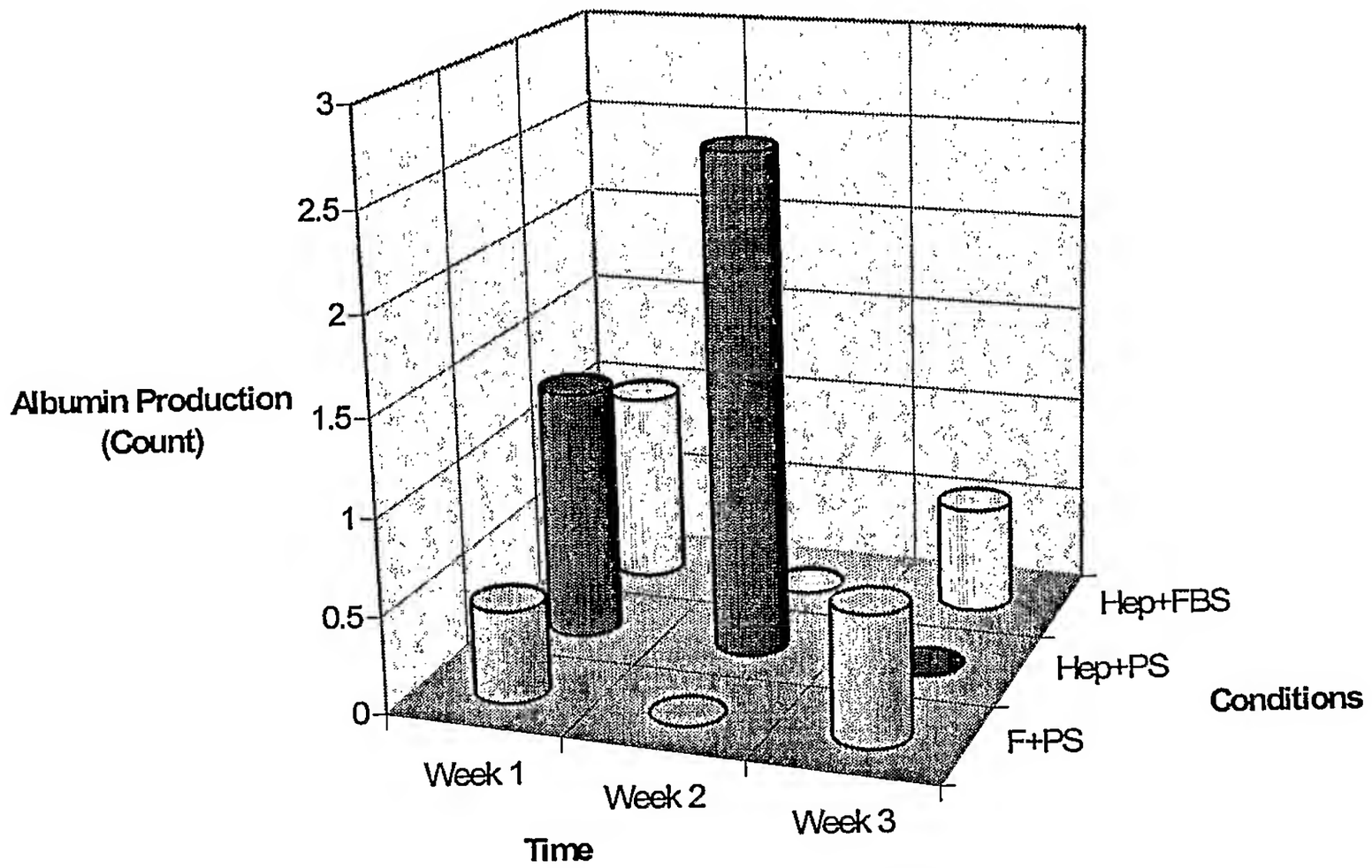

Count/Percentage
40
35
30
25
20
15
10
5
0
21.2
38.2
8.5
7.3
2.1
2.33
2 weeks in culture
3 weeks in culture
5 weeks in culture
albumin (in µg/ml) 21.2 2.33 38.2
Factor VIII (in %) 2.1 8.5 7.3
Time

Figure 1



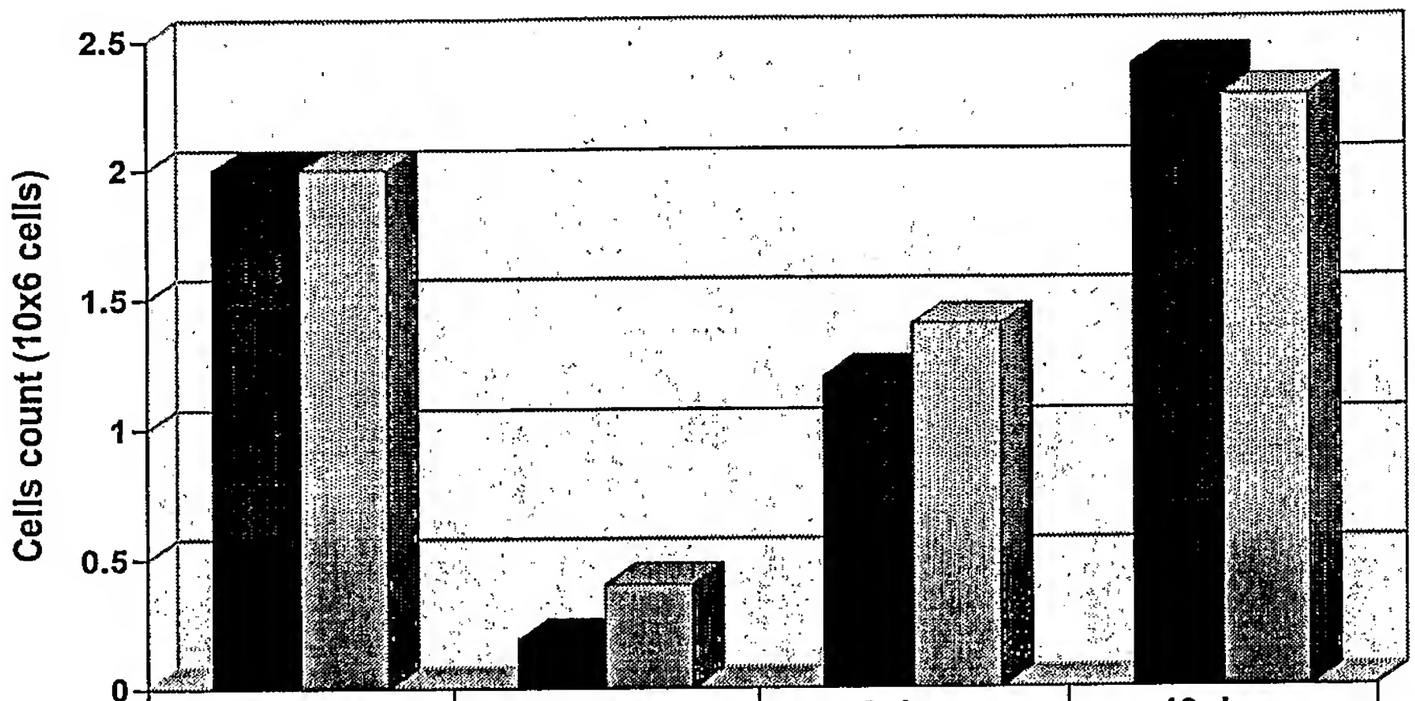
	Day 5	Day 7	Day 10	Day 13	Day 32	Day 54
ARF+FBS	1.3	0	0	0	2.9	0
ARF+PS	1.6	3.3	19.5	4.2	15.7	7.9
ARF NO S	3.9	0	0	0	0	0
COLL+PS	21.2	2.33	5.06	6.5	38.2	3.42
C+PS+F	2	1.2	2.48	1.16	3.76	0
C+PS+S	3.3	1.34	4.34	4.56	0.36	0

Figure 2



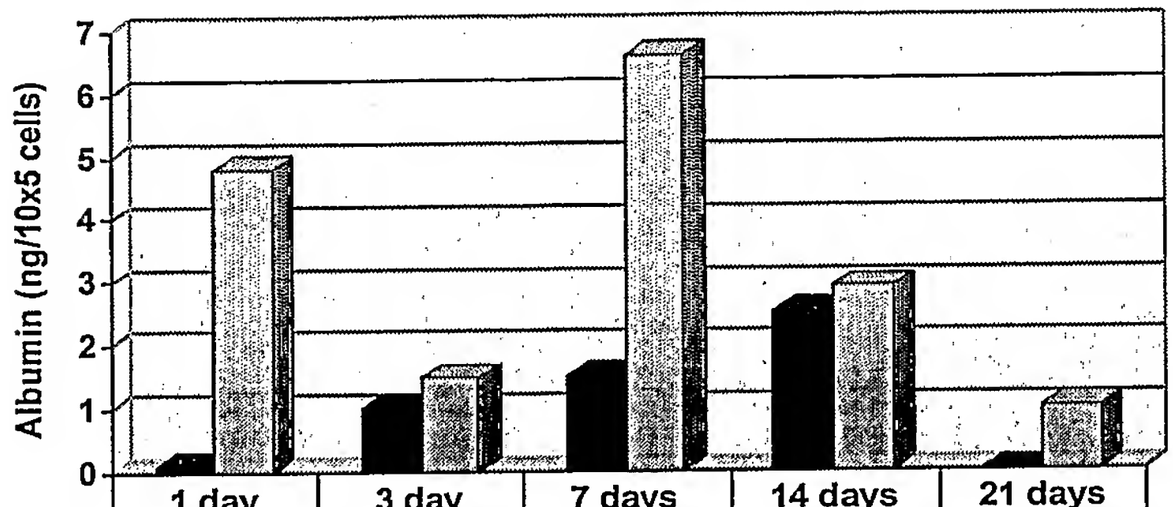
	Week 1	Week 2	Week 3
F+PS	0.48	0	0.67
Hep+PS	1.3	2.6	0
Hep+FBS	1	0	0.55

Figure 3



	1 day	2 days	6 days	13 days
Normal preparation	2	0.19	1.2	2.4
24 hours cold ischemia	2	0.4	1.4	2.28

Figure 4



	1 day	3 day	7 days	14 days	21 days
■ Routine isolation	0.095	1	1.53	2.5	0.03
▨ 24 hours cold ishemia	4.82	1.49	6.58	2.91	1

Figure 5

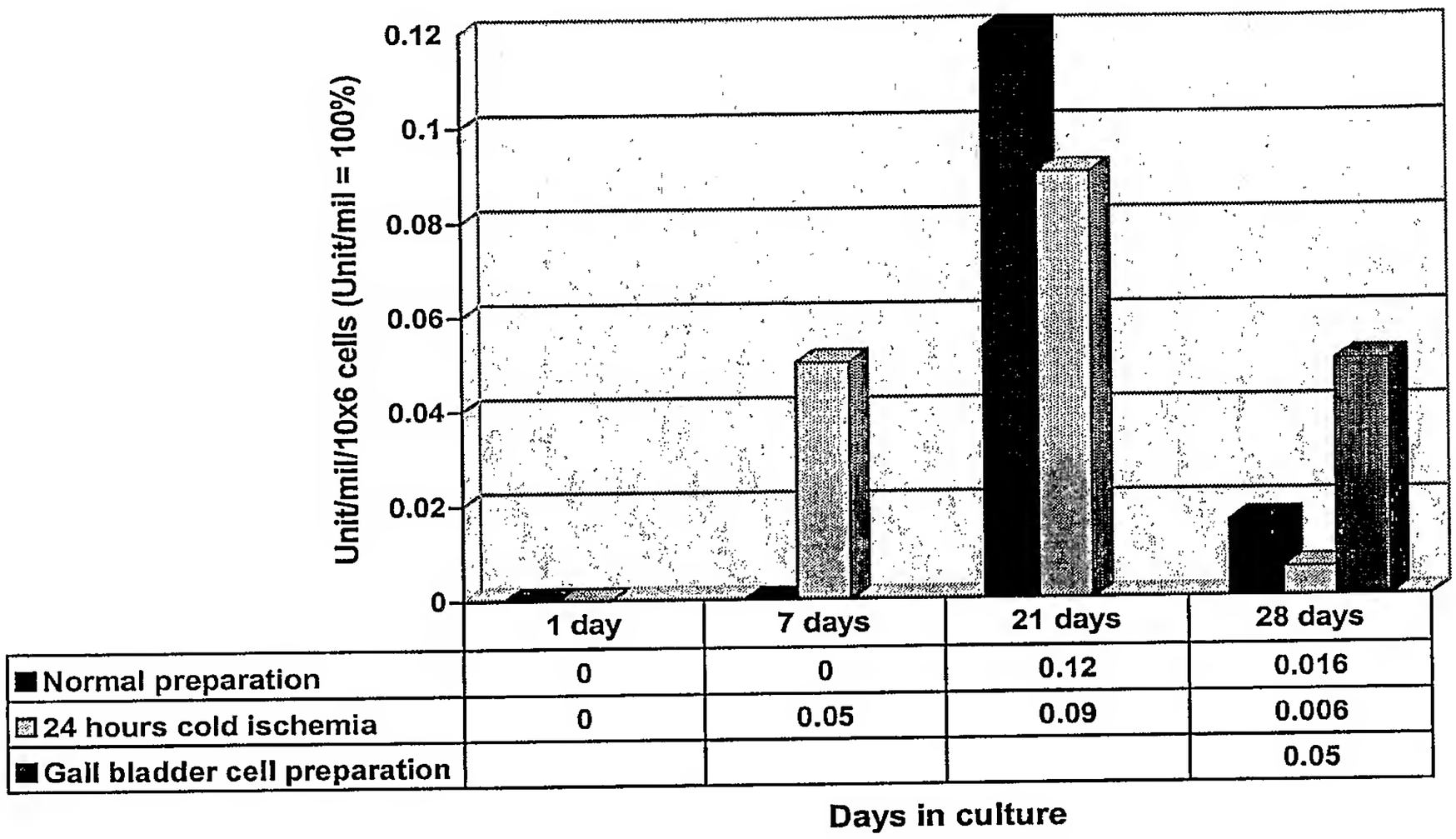

0.12
0.1
0.08
0.06
0.04
0.02
0
Unit/mil/10x6 cells (Unit/mil = 100%)
1 day
7 days
21 days
28 days
Normal preparation
0
0
0.12
0.016
24 hours cold ischemia
0
0.05
0.09
0.006
Gall bladder cell preparation
0.05
Days in culture

Figure 6

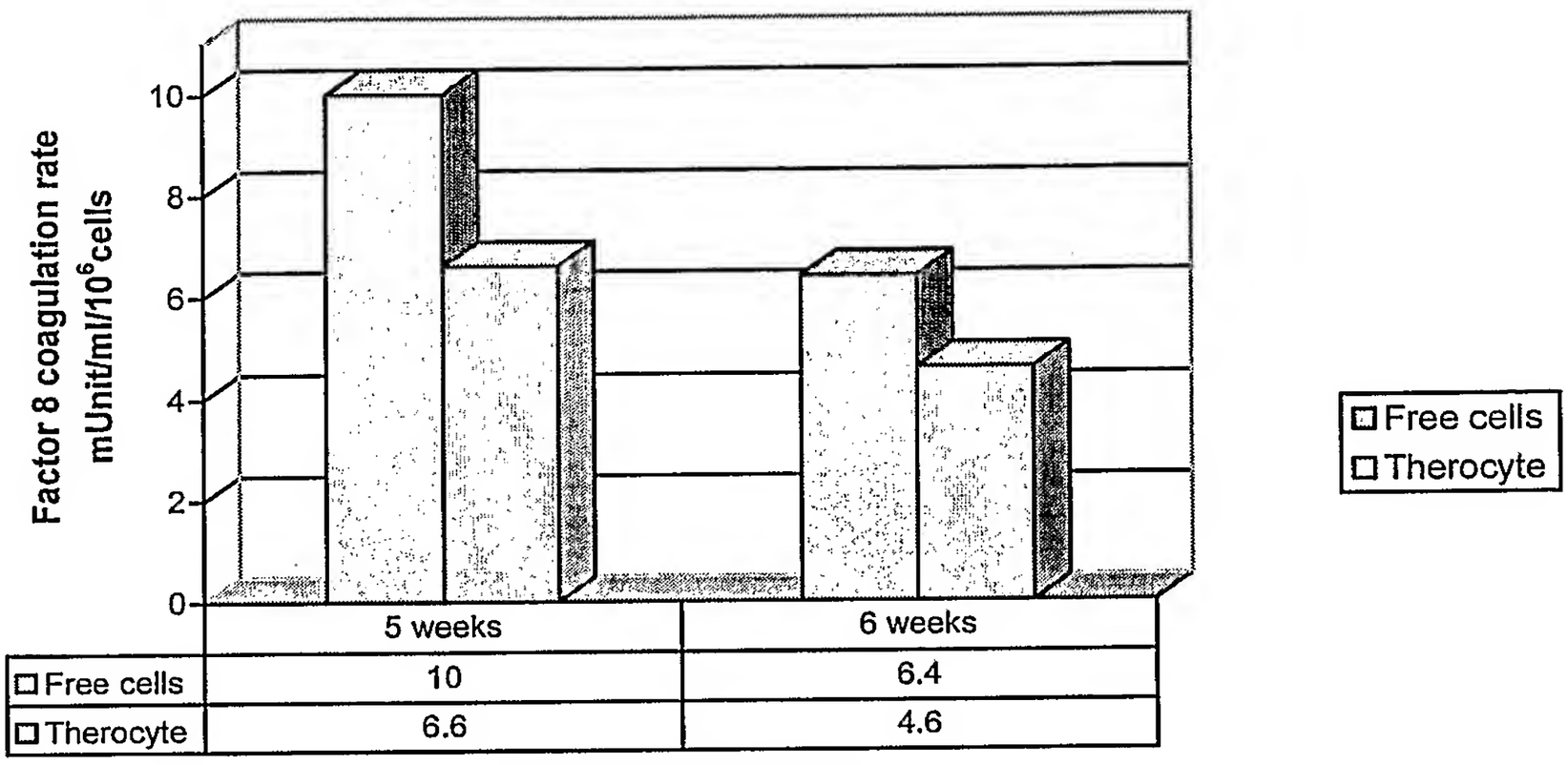

Factor 8 coagulation rate
mUnit/ml/10^6cells
10
8
6
4
2
0
Free cells
Therocyte
5 weeks
6 weeks
Free cells 10 6.4
Therocyte 6.6 4.6
Week post isolation

Figure 7

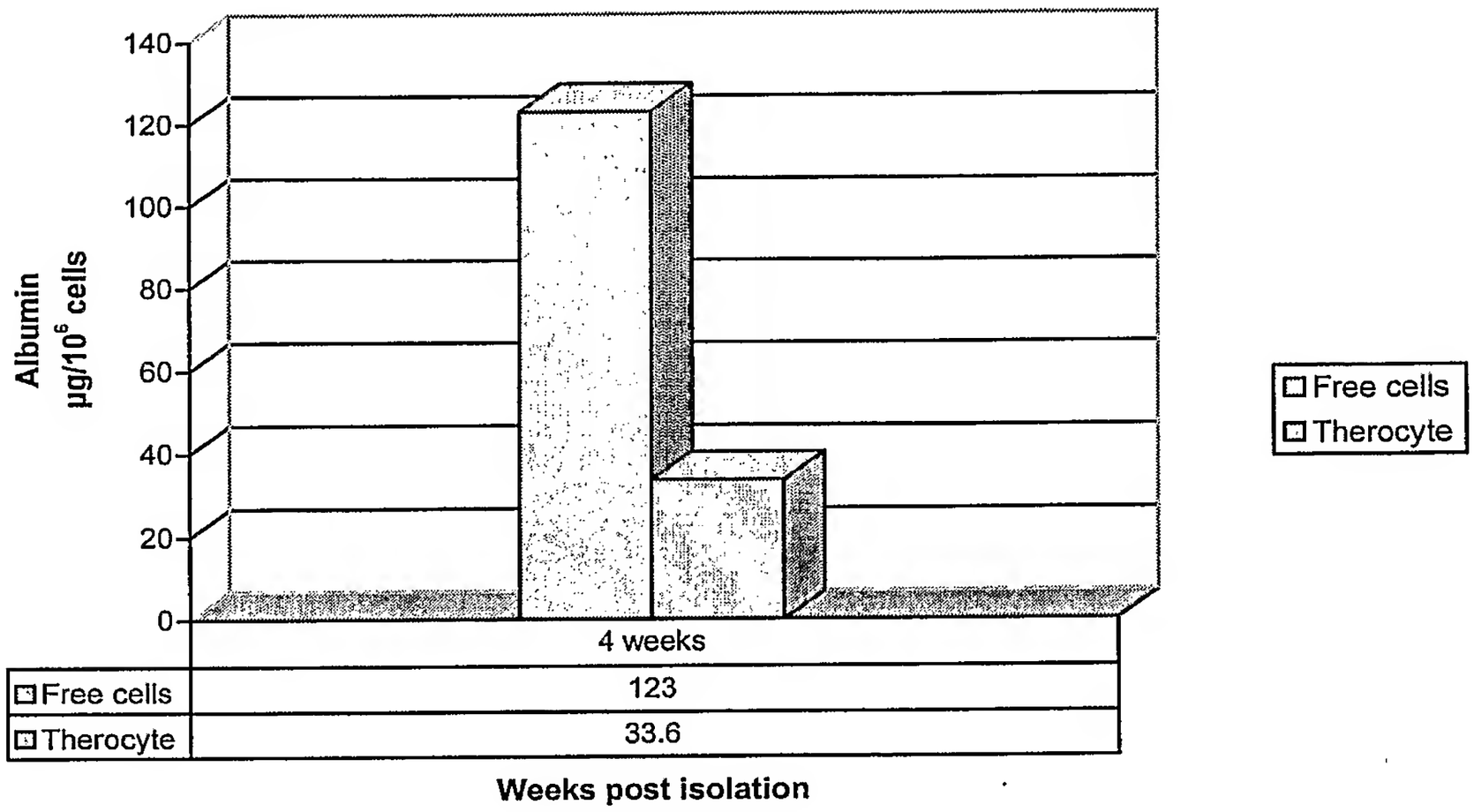

140
120
100
80
60
40
20
0
Albumin
µg/10^6 cells
Free cells
Therocyte
4 weeks
123
33.6
Weeks post isolation

Figure 8